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ESTUDO CLÍNICO E GENÉTICO DO PAPILOMAVÍRUS HUMANO SOROTIPO 16

MONIQUE FERRAZ DE SÁ BELTRÃO

RECIFE, PE

Maio 2011

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ESTUDO CLÍNICO E GENÉTICO DO PAPILOMAVÍRUS HUMANO SOROTIPO 16

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Biológicas, da Universidade Federal de Pernambuco, como parte dos requisitos exigidos para obtenção do título de Mestre em Ciências Biológicas.

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Dissertação de Mestrado apresentada pela aluna **MONIQUE FERRAZ DE SÁ BELTRÃO** ao Programa de Pós-Graduação em Ciências Biológicas, da Universidade Federal de Pernambuco, julgada adequada e **APROVADA**, pelos Membros da Banca Examinadora, na sua redação final, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas.

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Ela chegou nos anos 80
Na época da rainha dos baixinhos, parece que estou vendo
Dizer seu dia na escola: “meu pintinho amarelinho cabe aqui na minha mão..."
Amassar e colar bolinha de papel seguindo uma direção!!!!!
Ela chegou na era da informação tomando vulto, no tempo do super nitendo,
Se ouvia Triller e Cavaleiro do Zodiaco na Tv,
falava-se de solidadriedade no Criança Esperança e nas ferias via-se no cinema Super Xuxa contra baixo astral
Fim de semana o Ayrton Senna ser campeão de Formula I
Até se ouvir falar em globalização.
Teve seu Tamagoschi
Chegou o computador e o telefone celular
sabe que tivemos os” cara pintadas” e o impeachement de um presidente em nosso Brasil
que trocamos varias vezes nossa moeda, que os avós falavam em conto de rés
que a mãe falava em cruzeiros e que ela fala em Real.
Viveu a era do vinil ser substituido pelos CD e a chegada do DVD
Da Tv ganhar controle remoto, da chapinha.
Das cartas demoradas pra sua entrega serem substituidas pela informação imediata
da internet, do email, msn, orkut, twitter, ultrapassando oceanos.
Se fala em clonagem, e em projeto genoma humano
já alcança a época que os inibidores da protease do HIV alonga a vida dos pacientes
Falam em aquecimento global, noticiam a violência e o desequilibrio humano,
os acidentes,as perdas de grande comoção para a população
Ouve o pagode, axé e sertanejo e a musica eletronica tomar conta do campo musical
Com o fim da censura a TV aborda temas em suas novelas como homossexualidade, reforma agrária...
Vê o horror dos ataques terroristas de 11 de setembro em Nova York.
Ouve que o Brasil não deve mais não,
Brasil é credor da forte nação
Lula deixa o pais, sem pedintes pelo chão
Ela alcança um tempo de igualdade, Mulher Presidente, por que não?
Pensei tudo isso enquanto ouvia dissertar
sobre o PapilomaVirus para a bancada avaliar
é MESTRE mesmo no assunto,
diplomada com elogios para nova etapa alcançar.
Se conheço a ariana, o pé já está lá
permitindo a caminhada e descobertas fazer
pois sua missão é comprida, disse o Dr. para você.
Sou eu que agradeço os seus reconhecimentos
motivo de orgulho, por ter metas a alcançar
protetora com sua amizade e frases a aliviar
surge sempre como anjo
que sabe tão BEM GUARDAR.

(Maria de Lourdes Ferraz de Sá)

“O professor medíocre expõe. O bom professor explica. O ótimo professor demonstra.

O grande professor inspira.”

(William Arthur Ward)

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R E S U M O

O câncer cervical é o segundo tipo de neoplasia que mais acomete as mulheres no Brasil, com alta prevalência em Pernambuco. A associação desta patologia como o papilomavírus humano (HPV) já está bem estabelecida e atualmente, sabe-se que o HPV pode ser encontrado em vários outros locais de infecção, além da região genital. Com o intuito de apontar a distribuição corpórea do HPV pelo corpo humano foi realizada uma revisão da literatura buscando por sítios corpóreos em que o DNA viral já tinha sido identificado. Dentre os tipos virais de alto risco que mais acometem a população brasileira, sabe-se que o HPV16 aparece associado a mais de 50% dos achados. Baseado nisso, uma busca pela presença do DNA do HPV e pelos variantes virais do HPV16 foi realizada em mulheres de Pernambuco que apresentavam lesões genitais. Complementarmente, sabe-se que sistemas de expressão são amplamente utilizados para produção de diversas moléculas biológicas, sendo o bacteriano o mais rápido e fácil de ser utilizado. Visando melhor utilização do sistema de expressão em bactéria, desenvolvemos um método para detectar a produção de β-galactosidase em cepas heterólogas de *Escherichia coli*. Esse sistemas bacterianos vem sendo utilizados para produção de diversas moléculas virais, como oncoproteínas virais. Baseado no levantamento bibliográfico realizado foi possível identificar DNA viral nos mais diferentes sítios corpóreos, inclusive com ausência de lesões clínicas, apontando para a possibilidade do HPV agir como um oportunista. Da população estudada, mais de 50% foram positivas para o HPV, com achados de múltiplas infecções com tipos virais distintos, onde o variante Europeu foi o mais frequente nos casos de HPV16 positivo. A linhagem Origami (DE3) de *E.coli* demonstrou-se eficiente no ensaio colorimétrico expressando o gene da β-galactosidase com baixa produção de proteínas bacterianas. Baseado nisso, esse modelo bacteriano foi utilizado no processo de sub-clonagem do gene E7 do HPV16 permitindo após indução do promotor visualizar uma banda de 15 KDa na eletroforese de proteínas totais, banda provavelmente referente a oncoproteína viral. No entanto, faz-se necessário emprego de testes imunológicos com anticorpos específicos para confirmar sua produção e posterior purificação. A tipagem da população a respeito do variante do HPV mais predominante e produção da proteína E7 permitem o aumento nos conhecimentos dos diferentes mecanismos de interação do vírus com o hospedeiro e favorecem ao desenvolvimento de métodos diagnósticos e terapêuticos mais eficientes e específicos para as diferentes regiões do mundo.

Palavras-chave: Papilomavírus humano tipo 16; oncoproteína E7, câncer cervical; transformação celular; pRB, HPV.

A B S T R A C T

Cervical cancer is the second type of cancer that affects more women in Brazil, with high prevalence in Pernambuco. The association of this disease as human papillomavirus (HPV) is well established and currently, it is known that HPV can be found at other sites of infection, besides genital region. In order to highlight the distribution of HPV by the human body, we were a review of the literature searching for body sites where the viral DNA had been identified. Among the high-risk viral types that most affect the population, it is known that the HPV16 appears associated with more than 50% of the samples. Based on this, a search for the presence of HPV DNA and the HPV16 variants was performed in women of Pernambuco who had genital lesions. In addition, it is known that expression systems are widely used for production of various biological molecules, and the bacteria quickly and easy to use. For a better use of the expression system in bacteria, we developed a method to detect the production of β-galactosidase in heterologous strains of *Escherichia coli*. These bacterial systems have been used for production of several viral molecules such as viral oncoproteins. Based on the literature review was possible to identify viral DNA in many different body sites, including the absence of clinical lesions, suggesting to the possibility of HPV act as an opportunist. In the study population, over 50% were positive for HPV, with findings of multiple infections with different viral types, where the European variant was more frequent in cases of HPV16 positive. The strain Origami (DE3) *E.coli* was efficient in the colorimetric assay of the gene expressing β-galactosidase with low production of bacterial proteins. Based on this, this bacterial model was used in the process of sub-cloning of HPV16 E7 gene after induction of the promoter allowing a view of 15-kDa band on electrophoresis of proteins pool, band probably related to this viral oncoprotein. However, it is necessary to use immunoassays with specific antibodies to confirm its production and subsequent purification. Population classification on the most prevalent variant of the HPV and E7 protein production allow an increase in knowledge of the different mechanisms of interaction of the virus with the host and promote the development of diagnostic and therapeutic methods more efficient and specific for different regions of world.

Keywords: Human papillomavirus type 16; E7 oncoprotein; cervical cancer; cellular transformation; pRB

1. REVISÃO BIBLIOGRÁFICA

A família Papillomaviridae é formada porvírus de DNA circular, não-encapsulados com cerca de 8 kilobases. O DNA de Papilomavírus (PV) já foi isolado de lesões de pele de muitas espécies de mamíferos, aves e répteis. Muitos desses achados parecem apresentar o vírus como comensal da pele, onde um amplo espectro de genótipos já foram encontrados (Antonsson *et al.*, 2003; Antonsson *et al.*, 2002). Diversos PVs são capazes de infectar o homem, sendo estes denominados de papilomavírus humano (HPV) e alguns desses tipos são reconhecidos pela Organização Mundial de Saúde como carcinogênicos (IARC, 2007).

1.1. Paleovirologia

Os seres humanos têm provavelmente estado em contato com o HPV há milênios. As primeiras descrições de lesões associadas por desordem de relação sexual foram feitas com precisão pelo médico romano, Aurelius Celsus, no século 1 dC (Aufderheide, 2002). Nessa época, o médico já chamava os tubérculos anogenitais de "condilomas" e os tratava por cauterização com ferro em brasa (Hajdu, 2004). Múmias dos séculos 15 e 16, encontradas em Nápoles, Itália, também levaram a descoberta importantes resultados para paleopatologia e paleovirologia demonstrando a presença do DNA viral em tecido genital parafinado (Fornaciari *et al.*, 2003). O achado de HPV em relatos de séculos passados é útil para o fortalecimento da idéia sobre a evolução secular deste vírus.

1.2. Tipos e variantes virais

Existem aproximadamente mais de 100 genótipos do Papilomavírus capazes de infectar o ser humano; destes, 40 apresentam importância uroginecológica por serem capazes de infectar o trato anogenital (Munõz *et al.*, 2003). Os tipos virais podem ser classificados quanto ao risco de malignidade que oferecem ao trato genital em: alto-risco (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 e 82), e baixo risco, associado com lesões benignas genitais e verrugas na pele (HPV6, 11, 40, 42, 43, 44) (Dalstein *et al.*, 2009).O HPV16, assim como o tipo 18 são os genótipos virais mais prevalentes em carcinomas cervicais invasivos e neoplasias intraepiteliais cervicais, encontrados em mais de 50% dos achados (zur Hausen, 1996; Wheeler et , 2009; Vijayaraghavan et al. 2010).

Uma pequena proporção de lesões causadas por estes tipos virais irão progredir para um carcinoma cervical invasivo, o que indica o envolvimento de outros fatores, como a variação intratípica que ocorre na seqüência viral. Essa variação tem sido utilizada para estudar a

distribuição geográfica de HPVs, mas há evidências crescentes de que pode ser importante na determinação do risco de desenvolvimento de doença neoplásica (Giannoudis *et al*. 2001).

O genoma do HPV pode mudar por mutações pontuais, deleções e inserções, estas podem tornar-se permanentes numa população, se selecionadas positivamente ou, se forem funcionalmente neutros, pela expansão seletiva da população hospedeira infectada. Quando mutações nos oncogenes E6, E7 e L1 ocorrem com uma taxa de distância maior que 10% em comparação com outros tipos de HPV conhecidos, diz se que tem um novo tipo viral (de Villiers, 2004). Enquanto isso, variantes de um mesmo tipo viral diferem em 2% em regiões codificante e em 5% em regiões não codificantes em suas seqüências de nucleotídeos em comparação com a seqüência de referência (Bernard *et al.* 2010) (**Tabela 1**).

Tabela 1. Porcentagem de variações das sequências nucleotídicas do HPV originando novos tipos, subtipos e variantes.

	Variação da sequência nucleotídica
Novo tipo viral	> 10%
Novo subtipo viral	2-10%
Novo variante	2% na região codificante 5% na região não codificante

Variantes do HPV16 encontram-se agrupadas em diversos ramos, que foram nomeados de acordo com as suas principais distribuição geográfica. Atualmente, os seis principais ramos filogenéticos são: Europeu (Eu), Europeu-Asiático (Eu-As), Africano 1 (Af1) e Africano 2 (Af2), Asiático Americano (AA), e Norte Americano (NA) (Yamada *et al*. 1997).

As árvores filogenéticas podem ser construídas baseadas na análise comparativa das seqüências nucleotídicas ou da seqüência de aminoácidos e podem ser observadas como uma representação hipotética da história da evolução molecular, como pode se visualizar na **Figura 1.** A importância de conhecer a diversidade, taxonomia e evolução viral para o diagnóstico e abordagens terapêuticas têm sido valorizadas, visto que dados epidemiológicos e interações a nivel molecular têm sido apontadas como dependente destes fatores (Bernard *et al*. 2006).

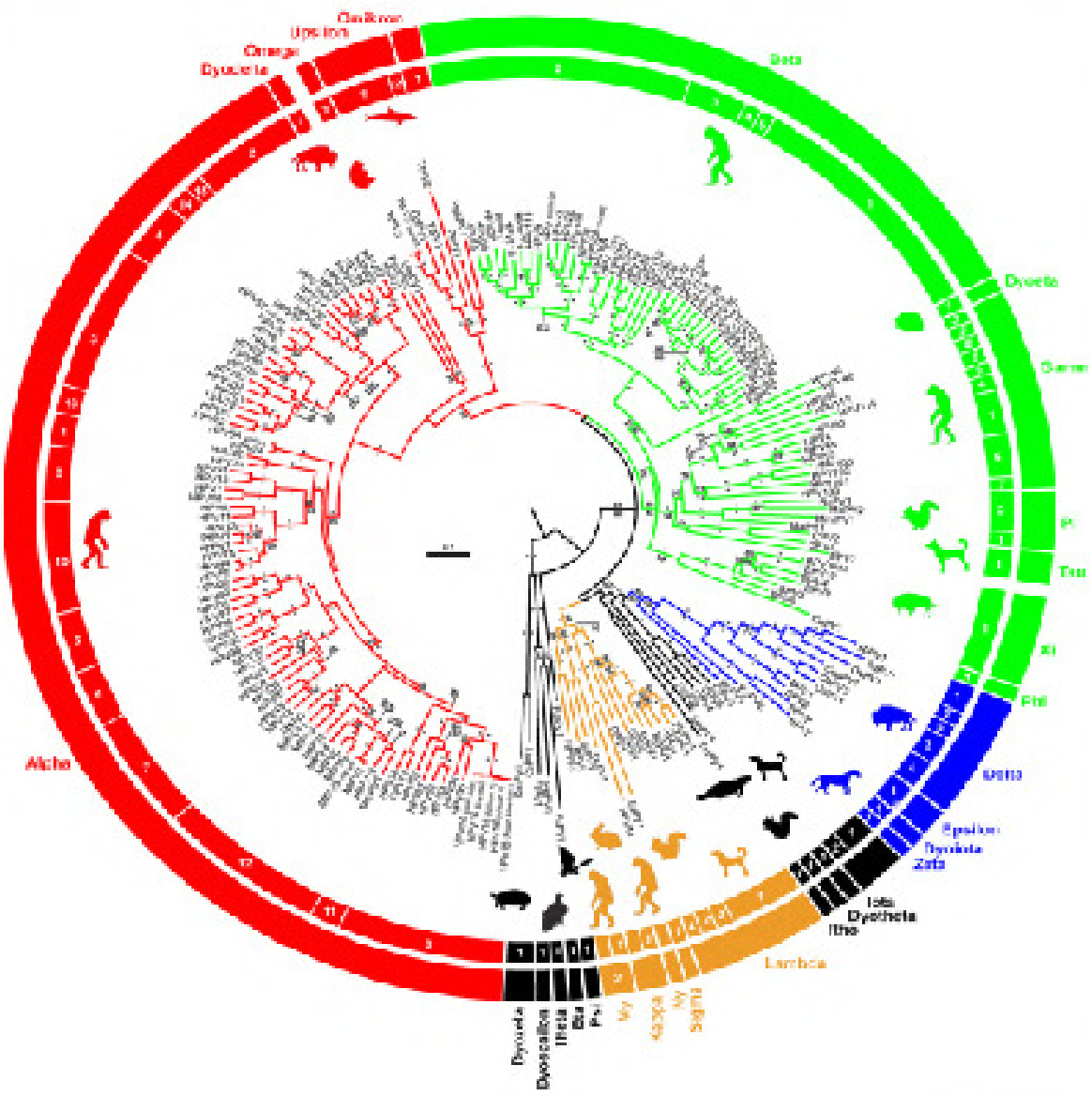

Figura 1. Árvore filogenética dos papilomavírus. A contrução foi realizada utilizando alinhamento de aminoácidos oriundo dos genes E1, E2 e L1 (extraída de Bravo *et al*., 2010).

O estudo de seqüências gênicas, como a realizada na busca por variantes virais predominantes em uma determinada população, pode viabilizar a aplicação de métodos profiláticos e terapêuticos, como vacinas tipo específicas de maneira mais prudente. Identificar o variante viral presente em uma população específica auxilia a esclarecer os motivos de maior resistência/susceptibilidade destas populações à infecção onde estão envolvidos aspectos genéticos, culturais, ambientais.

1.3. Genoma viral

O genoma do HPV (**Figura 2**) é formado por uma única molécula de DNA de dupla fita circular constituída por oito genes (L1 e L2 que codificam proteínas do capsídeo e E1, E2, E4, E5, E6 e E7 que codificam proteínas envolvidas na replicação, transcrição e transformação) e uma região não-codificante controladora (LCR) que contêm a sequência de origem de replicação (ORI) e a maioria dos promotores de transcrição (Terhune *et al.*, 2001).

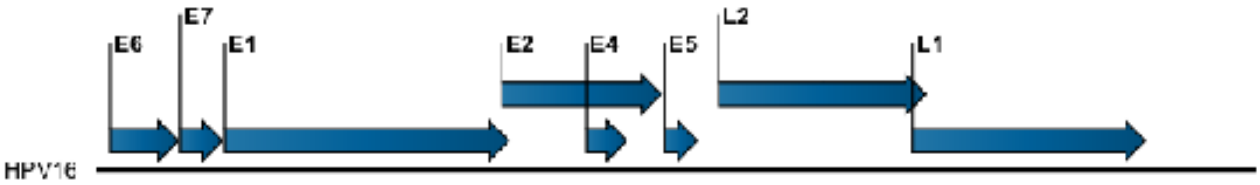

Figura 2. ORFs (*Open Reading Frames*) do HPV-16.

As ORFs E1, E2, L1 e L2 são particularmente bem conservadas entre os membros da família *Papovaviridae* (de Villiers, 2004). Na **Tabela 2** estão relacionadas as oito ORFs do HPV e suas principais funções.

O genoma do HPV pode replicar-se de forma episssomal extra-cromossômica em lesões benignas associadas aos HPVs de baixo risco, e em displasias leves e moderadas associadas aos HPVs de alto risco. Em geral, o desenvolvimento do câncer cervical está associado com a integração do DNA viral no genoma hospedeiro. No processo de integração, comum em casos de carcinoma cervical, o genoma circular viral é aberto entre os genes E1 e L1, levando à perda desse fragmento viral e inserção no genoma humano através desta região (Boshart *et al*. 1984; Baker *et al*. 1887). Sabe-se que este processo acarreta em um aumento da expressão dos oncogenes virais E6 e E7, apesar do mecanismo ainda ser desconhecidos (Garland, 2002).

1.4. Características dos oncogenes e oncoproteínas E6 e E7

Até hoje, acredita-se que as proteínas E6 e E7 não possuam atividade enzimática ou capacidade de se ligar ao DNA, mas apesar disso, são fundamentais para garantir as características de malignidade do Papilomavirus. O processo de transformação maligna das células infectadas pelo HPV é mediado através da interação entre os produtos dos genes virais

E6 e E7 e as suas oncoproteínas, que têm demonstrado serem suficientes para garantir a transformação, imortalização celular e formação do tumor (Munger *et al*. 1989; Doobar, 2005; Munõz *et al.*, 2006).

Tabela 2. Genes "Early", "Late" e região LCR codificados pelo papilomavírus humano, com seus respectivos tamanhos e funções.

Gene	Tamanho	Funções	Referências
E1	~ 1.940 pb	Codificar proteínas essenciais para replicação do DNA extracromossomal, complementação do ciclo viral e modular a transcrição de L1 e L2.	Motoyama *et al.*., 2004
E2	~ 1.090 pb	Codificar duas proteínas específicas responsáveis pela inibição e indução da transcrição das regiões "early".	Motoyama *et al.*., 2004
E4	~ 342 pb	Colapso da matriz do citoesqueleto (HPV-16), maturação viral.	Wilson *et al.*., 2007
E5	~ 237 pb	Interação com os receptores dos fatores de crescimento (EGF e PDGF), estimulando o crescimento celular.	zur Hausen *et al.*., 2002
E6	~ 500 pb	Desregulação do ciclo celular e degradação da p53.	zur Hausen *et al.*., 2002; Doorbar *et al.*.,, 2005
E7	~ 300 pb	Desregulação do ciclo celular e formação do complexo com a pRb.	Doorbar *et al.*., 2005; Motoyama *et al.*., 2004
L1	~ 1.500 pb	Formar o capsídeo viral maior.	Munõz *et al.*., 2006
L2	~ 1.400 pb	Formar o capsídeo viral menor.	Munõz *et al.*., 2006
LCR	~ 1000 pb	Elementos *cis* requeridos para expressão do gene, replicação do genoma, e empacotamento das partículas virais.	Munõz *et al.*., 2006

A oncoproteína E6 apresenta localização nuclear e possui aproximadamente 18 kDa (Moody *et al*., 2010), sendo capaz de interagir com várias proteínas relacionadas ao ciclo celular. A indução para fase S do ciclo celular de maneira não-programada e descontrolada normalmente leva a apoptose devido ativação da p53; entretanto a oncoproteína viral E6 liga-se a essa proteína supressora tumoral e a degrada por ubiquitinação proteossômica (Huibregtse *et al*., 1991), levando à resistência à apoptose. Além disso, a E6 também é capaz de interferir com a função da p53 ligando-se a duas histonas acetiltransferases, a proteína p300 e a proteína de ligação a CREB

(CBP), bloqueando a capacidade desses fatores de acetilarem a p53 e, portanto, aumentando a sua estabilidade (Patel *et al.*, 1999). A capacidade de ligação com outra histona, a acetiltransferase ADA3, que possui atividade similar a p53, direciona esta molécula a degradação. (Kumar *et al.*, 2002) Como também, ligando-se a proteína BAK (proteína pró-apoptose), fortalecendo o processo de resistência a apoptose e ao aumento da instabilidade cromossomal (zur Hausen, 2002).

Embora os efeitos da oncoproteína E6 de alto risco sobre a p53 sejam fundamentais para o desenvolvimento de câncer genital, rotas alternativas (**Figura 3**) independente da p53 desempenham papéis igualmente importantes (Moody *et al.*, 2010). Linhagens celulares deficientes ou com o gene E6 mutante para a degradação de p53 podem ainda imortalizar células (Kiyono *et al.*, 1998). Estes dados sugerem que as interações com outros fatores celulares são necessárias para o desenvolvimento do câncer, tais como as proteínas PDZ, associadas apenas com proteínas E6 de alto risco (Thomas, 2008). Estudos com camundongos transgênicos, "desligando" o domínio PDZ de ligação a E6, demonstraram ausência de desenvolvimento hiperplásico (Nguyen *et al.*, 2003).

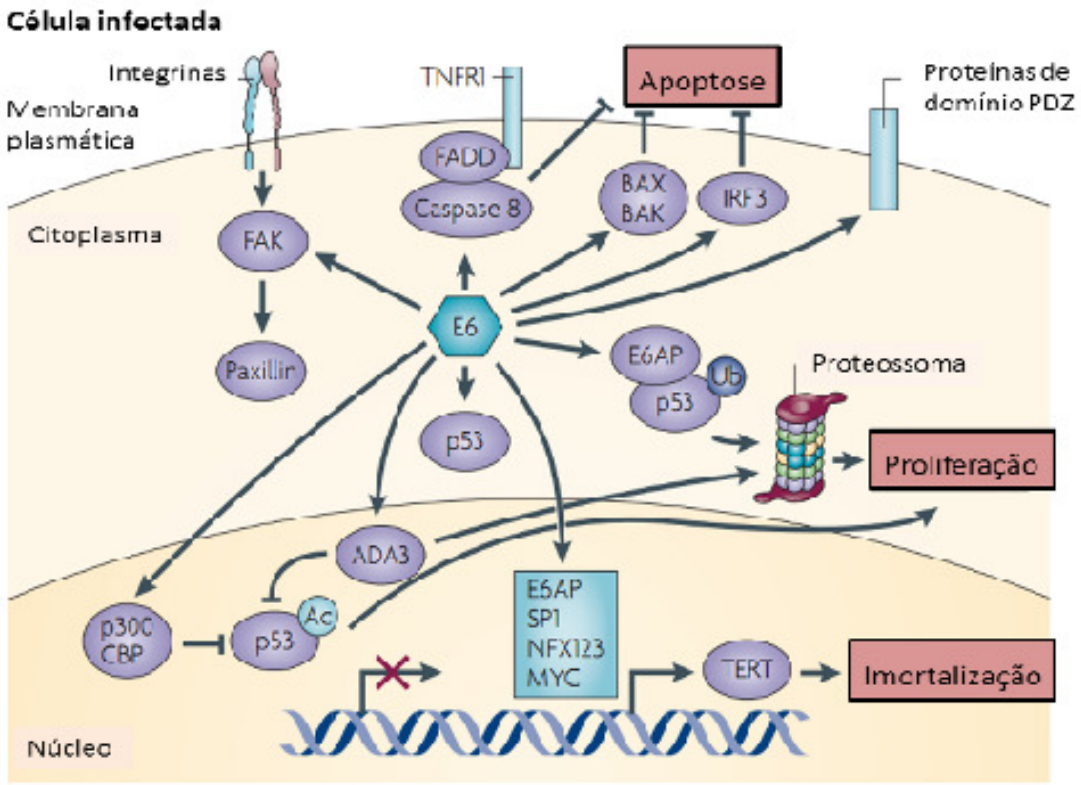

Figura 3. Proteínas celulares e rota metabólica afetada pela oncoproteína E6 (modificado a partir de Moody et al. 2010).

A proteína E7 contribui para a imortalização celular devido à sua capacidade de interagir com a proteína supressora tumoral do retinoblastoma (pRB), assim como membros de sua família, a p107 e p130. A família do Rb controla a transição de fase G1 para S, regulando a atividade da família de fatores de transcrição E2F (Dyson *et al.* 1998). Em células normais, a pRB reprime a transcrição depromotores E2F-dependente diretamente pela ligação ao domínio de transativação da E2F recrutando vários modificadores de cromatina, tais como histonas desacetilases (HDACs) (Harbour *et al.* 2000). No final da fase G1, pRB é fosforilada por complexos ciclina dependente de quinase (CDK), o que resultana dissociação dapRB de E2F, levando à transcriçãoda fase S. A ligação da E7 de alto risco a pRB interrompe o complexo RB-E2F, resultando naexpressão constitutiva de genes E2F-responsivos, como ciclina A e ciclina E, promoção da fase S precocemente e de síntese de DNA (Zerfass et al. 1995). Além de formar complexos com a pRB, a E7 também a orienta a degradação através da via proteossômica ubiquitina-dependente (Boyer et al. 1996) induzindo a anti-apoptose. A interação E7-pRB-HDAC (**Figura 4**) é essencial para a manutenção viral e para a manutenção de um ambiente de multiplicação celular, portanto, necessário para replicação viral.

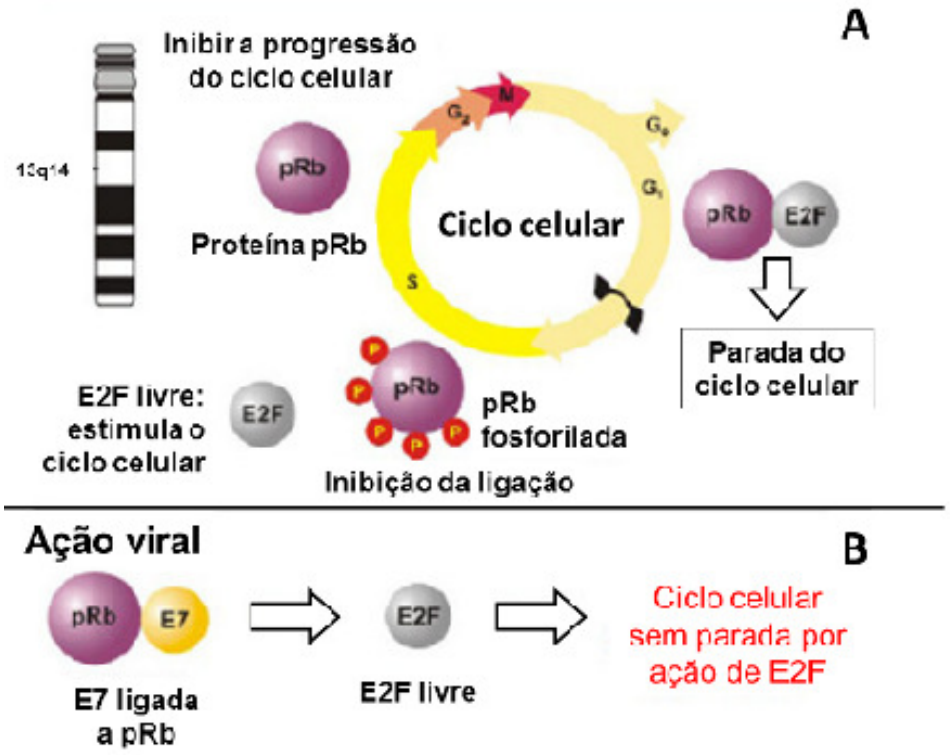

Figura 4. Esquema de interferência da E7 no ciclo celular. (A) Associação de pRB com o fator E2F e consequentemente regulação por inibição do ciclo celular. (B) Complexo E7-pRB que resulta na liberação do fator E2F e proliferação descontrolada celular (Silva et al., 2003).

Além da interação conhecida com a pRB, a E7 é uma potente inibidora da atividade da p21 e p27, inibidores de quinase ciclina-dependentes, evitando o controle de checagem da fase G1 (Jones *et al*., 1997) por estimular indiretamente as ciclinas e através da ativação direta de quinase ciclina-dependente 2 (CDK2). A atividade CDK2 é capaz de estimular ativação da telomerase e amplificação centrossomal, aumentando e interagindo com γ-tubulina, conduzindo a um risco maior de instabilidade genômica; além de induzir danos ao DNA pela ativação da via ATM-ATR levando ao acúmulo de alterações cromossômicas (Moody *et al*., 2010).

A interação da E7 com moléculas humanas também garante a capacidade viral de escapar ao controle do sistema imunológico devido à sua ligação com componentes da resposta ao interferon (IFN) (fator de regulação 1 do IFN - IRF1 e p48). Tal acoplamento estimula a formação de um complexo que bloqueia a translocação do heterodímero STAT1-STAT2 para o núcleo em resposta ao IFN (Park et al., 2000). O bloqueio da dimerização dos STATs impede a formação de complexo cujo resultado seria a ativação da transcrição de genes estimuladores do interferon, e consequentemente oimpedimento da replicação viral. A interação da E7 com essas proteínas citadas resultam em instabilidade genômica, proliferação e apoptose (Moody *et al*., 2010) (**Figura 5**).

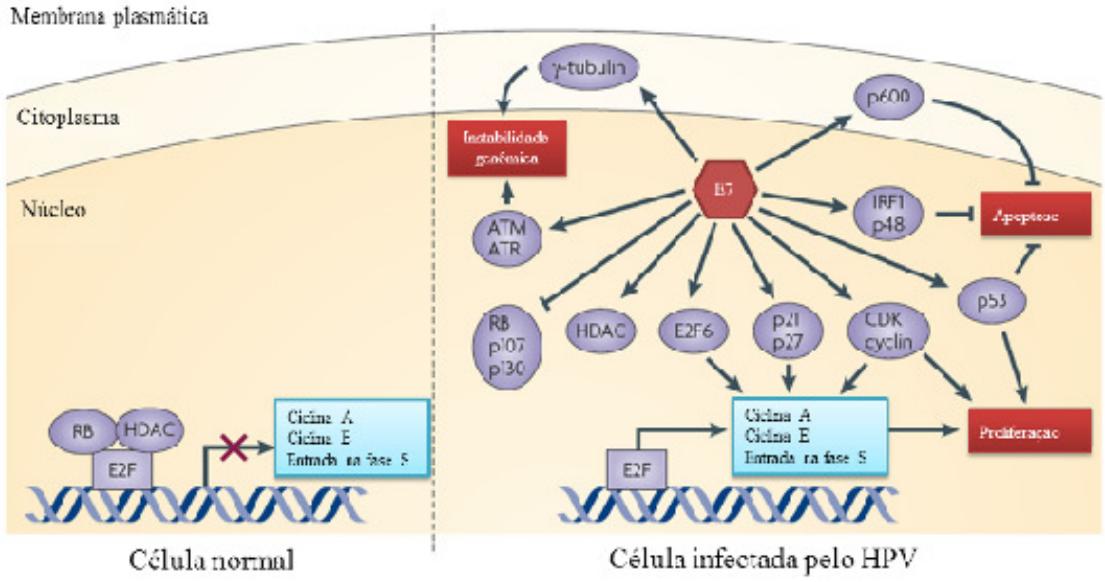

Figura 5. Interações da oncoproteína E7 do papilomavírus humano com proteínas celulares do organismo infectado (modificado a partir de Moody et al., 2010).

De acordo com o tipo de célula e o tipo viral, a oncoproteína E7 do HPV também pode inibir a apoptose e causar uma diminuição dasensibilidade à morte celular mediada por citocinas. Estudos realizados por Thompson e colaboradores (2001) demosntraram que a oncoproteína E7 do HPV tipo16 inibe a apoptose mediada pelo fator de necrose tumoral tipo α (TNFα) e ativação de caspase-8, em fibroblastos humanos normais.

1.5. Infecção pelo HPV

A infecção pelo HPV é o fator de risco mais importante na etiopatogênese do câncer de colo de útero, no entanto apresenta um padrão transitório onde apenas 10% das mulheres infectadas por tipos de alto-risco irão desenvolver a doença (Stanley *et al*., 2007). Conforme variáveis pertinentes ao vírus e ao indivíduo, o período de incubação da infecção pode variar de semanas até anos, onde comumente se manifesta entre duas semanas a oito meses (Ito, 2010). Em alguns casos em que não foi realizado tratamento, foi demonstrado que o vírus é eliminado devido à ação do sistema imune, em um período de 12 a 24 meses após o diagnóstico inicial (Ho *et al*., 1998).

Para entender como a infecção pelo HPV causa resultados malignos, é importante descrever o ciclo de vida destes vírus (**Figura 6**). O HPV infecta os queratinócitos na camada basal do epitélio, que se torna exposta através de lesões. No início da infecção, o genoma viral apresenta-se na forma episssomal onde a multiplicação viral é baixa e os genes virais precoces (*early*) são expressos. À medida que o epitélio vai se diferenciando, o vírus faz uso da replicação do DNA celular, multiplicando conjuntamete. A expressão dos genes E6 e E7 desregulam o ciclo celular, levando a diferenciação das células em fase S, que permite a amplificação do genoma viral nas células que normalmente teriam saído do ciclo celular. A fase final produz as proteínas capsidiais L1 e L2 que protegem o genoma viral recém sintetizado. Nesta fase, o vírus agora é liberado nas camadas mais superficiais do epitélio podendo infectar novos queratinócitos (Cheng *et al.*, 1995).

1.6. Fatores de risco no câncer cervical

O fator de risco mais importante para o desenvolvimento do câncer do colo do útero é a infecção genital pelo papilomavírus humano. No entanto, fatores predisponentes são fundamentais para evolução ou regressão da patologia.

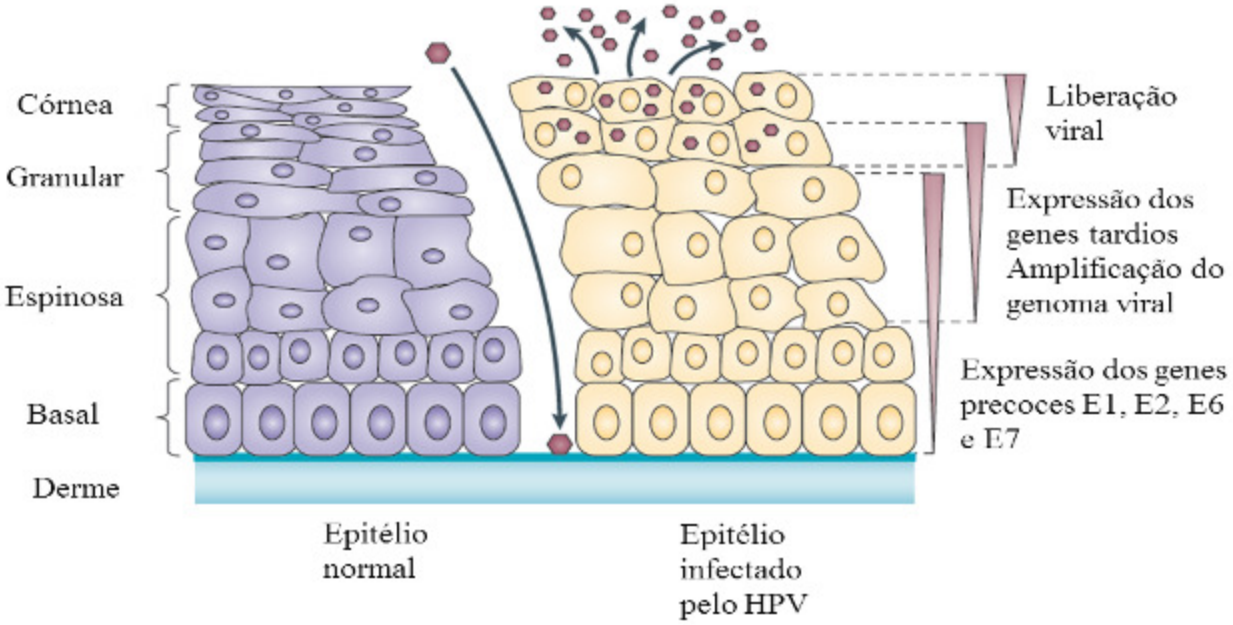

Figura 6. Esquema comparativo do epitélio normal e infectado pelo Papilomavirus humano (modificado a partir de Moody et al. 2010).

Park e Kang (2000) demonstraram que as proteínas E6 e E7 dos HPVs de alto risco são responsáveis por gerar grande instabilidade genética, aumentando as taxas de mutações espontâneas em presença de carcinógenos como o derivado do tabaco, MNNG (N-Metil-N'-nitro-N-nitrosoguanidina).

Potenciais fatores de risco associados ao desenvolvimento do câncer cervical podem ser divididos em três grupos: (1) cofatores ambientais ou exógenos, incluindo o uso de anticoncepcionais orais, tabagismo, e co-infecção pelo HIV ou outros agentes sexualmente transmissíveis; (2) cofatores virais, tais como infecção por determinados tipos de HPV, carga viral, integração viral e co-infecção com mais deum tipo de HPV; (3) cofatores do hospedeiro: multiparidade, hormônios endógenos, resposta imunológica e susceptibilidade genética (Von Linsingen, 2008).

1.7. Classificação do achado citológico

De acordo com a fase que se encontre a infecção viral, o diagnóstico para o HPV pode ser realizado de diferentes maneiras. Na fase aguda, a análise é feita observando as manifestações clínicas: condiloma acuminado (verrugas); na fase latente, é conveniente utilizar as técnicas de diagnóstico molecular – PCR e na fase sub-clínica faz-se colposcopia e microscopia dos

raspados vaginais em busca de células apresentando alterações causadas pelo vírus (Gompel *et al.*, 1997).

Por conta da subjetividade e variedade dos laudos citopatológicos para diagnóstico de células infectadas pelo HPV, estudiosos vem buscando padronizar a classificação das lesões durante reuniões organizadas pela Sociedade Brasileira de Citopatologia (SBC). A primeira normatização existente foi registrada em 1941 por George Papanicolaou, a qual foi modificada na reunião de 1988 da SBC pela Classificação de Bethesda. Em 1991, esta classificação foi revisada e em 2001 introduziram-se novas técnicas para detecção do HPV em achados citológicos, passando a ser conhecido por sistema NIC (Frappart *et al.*, 2006). Na **Tabela 3**, é possível observar essas classificações, as substituições de nomenclatura e as orientações clínicas. A maioria das lesões intra-epitelial de baixo grau (NIC-1) poderia evoluir para o desenvolvimento de um câncer cervical, no entanto, aproximadamente 90% dos casos regridem em 12 a 36 meses sendo eliminado pelo sistema imunológico (Syrjanen *et al.*,1996), ocorrendo regressão natural. Os achados citopatológicos de NIC-1 são caracterizados por células superficiais e intermediárias apresentando alterações morfológicas, presença de coilócitos (**Figura 7**) – células com cavitação perinuclear com núcleos volumosos, cromatina borrada, bi oumultinucleação (Gompel *et al.*, 1997). Caso a infecção persista, pode ocorrer à evolução desta lesão inicial para um NIC-2 e até um carcinoma escamoso.

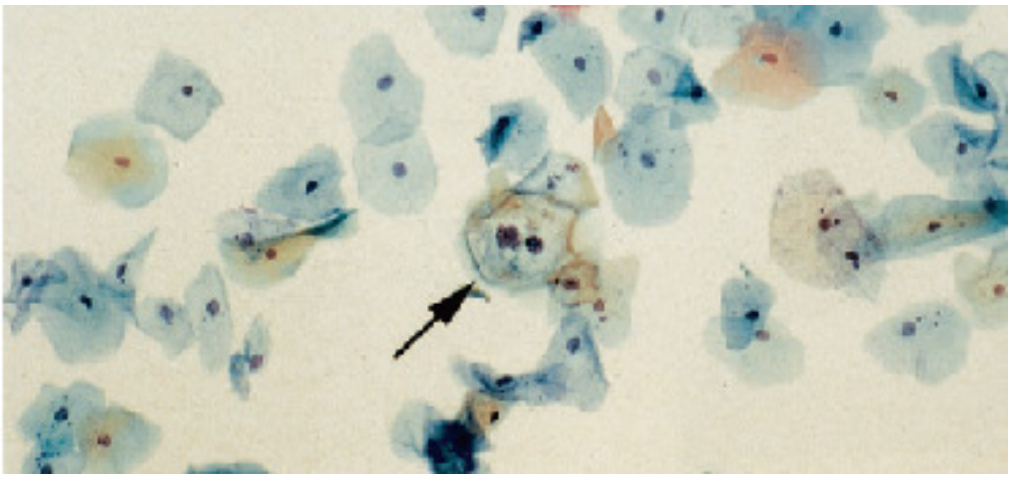

Figura 7. Microscopia óptica demonstrando alterações morfológicas celulares causadas pelo HPV – Coilócitos (Burd *et al.*, 2003).

Tabela 3. Classificação de Papanicolau, Bethesda e NIC para displasias de células escamosas cervicais – Interpretações e orientações. (modificado a partir de Burd et al. 2003) e http://www.prevencaodecancer.com.br

Classificação			**Interpretação**	**Orientação**
Papanicolau	***Sistema Bethesda***	***Sistema NIC***		
Classe I	Negativo para células neoplásicas	Normal	Normal	Repetir exame em 1 ano ou conforme orientação médica.
Classe II	Inflamatório		Característico da 2ª fase do ciclo ou corrimento	Repetir exame em 1 ano ou conforme orientação médica e tratar inflamação se necessário.
Atipia celular escamosa	ASCUS - atipias de células escamosas de caráter desconhecido		Leve suspeita de alteração	Colposcopia e biópsia dirigida definirão o tratamento
Atipia celular glandular	AGUS - células glandulares atípicas	NIC 1	Suspeita de alteração	Colposcopia e a biópsia dirigida que definirá o tratamento. Em caso de menopausa, investigar o endométrio.
Classe III	LSIL - lesão intra-epitelial de baixo grau	NIC 2/ 3	Alterado	Colposcopia e biópsia dirigida definirão o tratamento
	HSIL - lesão intra-epitelial de alto grau		Alterado	Idem
Classe IV	HSIL - lesão de alto grau		Alterado	Idem
Classe V	Suspeita de câncer	Carcinoma celular escamoso invasivo, Adenocarcinoma	Alterado	Idem

1.8. Papilomavírus humano versus Câncer do Colo do Útero

No final dos anos 60, a neoplasia cervical eraassociadacom a presença do herpes vírus tipo II (Rawls *et al.*, 1968). Só no começo da década de 70, as associações do câncer cervical com o HPV foram confirmados (zur Hausen, 1974). O pesquisador alemão e virologista Harold zur Hausen, vem conduzindosuaspesquisas nesta área por muitos anos e fazcontribuições até hoje, principalmente relacionados com a associação do vírus com as lesões causados no homem[1]. Atualmente, sabe-se que o HPV pode ser encontrado também em câncer de pênis, vagina, vulva, ânus, região perianal e cabeça e pescoço (zur Hausen, 2009a; 2009b), sítios de infecção diferentes ao qual foi originalmente vinculado (zur Hausen *et al*., 1977; Shukla *et al*., 2009).

[1] Em 2008, esta associação foi premiada com o Prêmio Nobel de Medicina (http://nobelprize.org/nobel_prizes/medicine/laureates/2008/).

Mundialmente, a neoplasia do colo do útero acomete 500 mil casos novos por ano, sendo responsável pelo óbito de aproximadamente 230 mil mulheres. Este câncer é o segundo tipo mais incidente em mulheres nas quatro regiões pesquisadas (Brasil, Nordeste, Pernambuco e Recife). A infecção pelo HPV no colo uterino, quando descoberto no estágio inicial, apresenta excelente prognóstico, ou seja, tem ampla possibilidade de cura. No entanto, quando há o diagnóstico tardio, as taxas de mortalidade são altas. No Brasil, visando alterar esse perfil de morbidade e mortalidade feminina por câncer de colo uterino (**Figura 8**), o Ministério da Saúde criou, em 1997, o Programa Viva Mulher. A forma de abordagem adotada pelo Programa é o oferecimento do exame citopatológico às mulheres de maior chance para o desenvolvimento de câncer do colo do útero e suas lesões precursoras, além do tratamento ou acompanhamento das lesões detectadas.

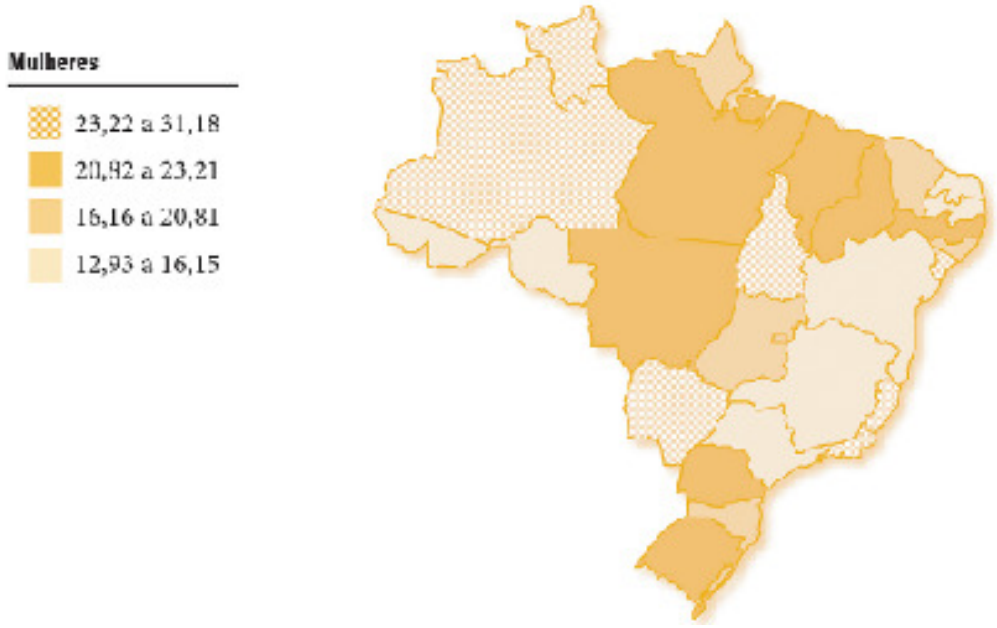

Figura 8. Representação espacial das taxas brutas de incidência de neoplasia maligna do colo do útero por 100 mil mulheres (INCA, 2010).

O tratamento priorizado para o câncer cervical é em nível ambulatorial, por meio da cirurgia de alta freqüência (CAF) orientada pela colposcopia (Brasil, 2001). Hoje, o programa busca integrar todos os dados coletados ao longo dos anos, desde sua implementação com o Sistema de Informação do Câncer do Colo do Útero (SisColo) integrado com outros sistemas de dados como, os do DATASUS. Este sistema é útil como um indicador, revelando o alcance das mulheres da população estudada através da oferta de exames (Ministério da Saúde, 2010).

No Brasil, desde 2006, estão registradas pela Agência Nacional de Vigilância Sanitária (Anvisa/MS) duas vacinas profiláticas: a Gardasil e a Cervarix. A primeira imuniza contra quatro

tipos virais: HPV 6, 11, 16 e 18, enquanto que a segunda tem ação sobre os dois tipos mais malignos, o 16 e 18. As duas vacinas são baseadas na injeção de particulas semelhantes aos virus (VLPs), no entanto com ausência de DNA viral responsável pela sua virulência (Stantey, 2007). A incorporação da vacina contra HPV no Programa Nacional de Imunizações está em discussão pelo Ministério da Saúde e pode se constituir, no futuro, uma importante ferramenta no controle do câncer do colo do útero.

2. JUSTIFICATIVA

O câncer é um grande desafio científico no que se refere a sua compreensão biológica, prevenção e controle. O HPV16 e o 18 são os tipos de HPV mais comumente envolvidos na malignização de lesões cervicais que levam ao câncer, sendo conjuntamente responsáveis por mais de 70% dos casos. Este trabalho foi realizado com o intuito de contribuir para um maior conhecimento acerca da compreensão do mecanismo de ação viral, utilizando busca de variantes e localização dos possíveis sítios de infecção viral. O vírus é capaz de infectar diversos sítios corpóreos, sendo apontado como possível causador de outros tipos de cânceres além do cervical. O tipo de variante encontrado numa determinada população pode resultar num fator de risco adicional para a infecção em diferentes sítios do corpo e além do desenvolvimento de carcinoma invasivo do colo do útero. O conhecimento adquirido com esse trabalho poderá ser útil para avaliação da evolução e história natural das neoplasias provocadas pela infecção pelo Papilomavírus humano.

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar a abrangência da infecção pelo Papilomavírus Humano em diferentes sítios do corpo, observando a presença dos diferentes tipos virais e variantes do HPV16 em amostras de lesões genitais femininas, alem de desenvolver um sistema de expressão que permita produção e purificação eficiente da oncoproteína E7 viral com intuito de ampliar o conhecimento sobre sua importância na malignidade viral.

3.2. Objetivos Específicos

- Prospectar possíveis sítios de infecção onde o HPV já foi isolado no corpo humano;

- Selecionar um sistema bacteriano para expressão de proteínas heterólogas eficiente;

- Identificar a presença do HPV em amostras coletadas de lesões genitais;

- Identificar os tipos de HPV16 variantes mais frequentes na população estudada;

- Identificar diferentes SNPs nos genes E6 e E7 dos variantes de HPV16 obtidos de amostras clínicas;

- Sub-clonar o oncogene E7 do HPV16 em sistema de expressão bacteriano.

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5. CAPÍTULO I

1. TÍTULO: Human Papillomavirus Infection thought the Human Body (review)

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HUMAN PAPILLOMAVIRUS INFECTION THROUGH THE HUMAN BODY

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ABSTRACT

Human papillomavirus (HPV) is currently a clinically important virus in view of the highest number of lesions that it causes at different sites of the human body. The diagnosis of HPV infection should be made based on visual inspection of characteristic clinical signs and laboratory tests. Organs of the genital tract are more common sites where this virus can be found, but with the increasing the sensitivity of diagnostic technique, it is possible to identify viral presence in different regions of the body such as tissues of the digestive, respiratory and urinary tracts. These findings demonstrate that HPV is able to infect various cells, tissues and organs. The widespread presence of the virus in the human body, often in latent form, led us to consider the hypothesis that human papilloma virus latency may be associated with no disease, which opens questions about the chance of this virus not causing disease in specific sites of the human body, but rather behaving like a symbiotic microorganism.

Keywords: *Human papillomavirus*; viral dissemination; neoplasia; condyloma; warts; cervical cancer.

INTRODUCTION

Viral infections, such as those caused by HIV, HCV, Ebola, and others constitute a public health problem worldwide, especially in developing countries. Papillomaviruses (PVs) are currently represented by 189 genotypes of which 120 were isolated from humans[1]. They are divided into 16 PVs genera[2], and alpha-papillomavirus is considered the most clinically important genus as it includes the largest number of HPV that cause lesions. HPV types can be broadly grouped according to the degree of cell damage that they are able to cause, resulting in high or low risk[3]. Additionally, they can be described as cutaneous types or mucosotropic types based on their tissue tropism[4]. The aim of this review is to give an overview of HPV infection sites in the human body beyond the genital tract. Moreover, the 'infective' nature of this virus needs elucidation as human papillomavirus (HPV) can establish latent residence in normal epithelia and in lymphocytes without clinical sign of disease[5].

VIRUS BIOLOGY

The HPV genome consists of double-stranded circular DNA of about 8 kb comprising three regions. The non-coding region is called the Long Control Region (LCR), containing the viral origin of replication (ORI) and most of the transcription promoters. Two genes are in a region designated Late (L1 and L2), since they are the last genes to be expressed[6]. They encode structural proteins and facilitate the release of virions by the formation of a viral capsid[7]. Most viral genes are located in the early region (Early), which is expressed at the beginning of the viral cycle. Proteins E5, E6 and E7 are classified as oncogenic because they are involved in malignant transformation of HPV-infected cells[8]. E1 and E2 are known as regulatory proteins, while E4 protein is responsible for viral maturation and altering the intracellular matrix. In high-grade intraepithelial cancers, E1/E2 genes are depleted/interrupted[9].

The information obtained about the life cycle of papillomaviruses comes from natural infections in animals, including rabbits, cattle and dogs[10], since in vitro replication of papillomavirus are limited due to the difficulty in reproducing the stratification necessary for cellular viral maturation. Human infection with HPV is most focused in

cervical cancer; the first site in the body in that HPV was found[11]. The infection usually begins with the entry of the virus into cells of the basal layer of the epithelium (10 viral copies/cell). When they reach 50 copies/cell, the genes E1, E2, E6 and E7 begin to be expressed, leading to viral replication with low expression of these genes, in parallel with cell replication. The amplification of viral copy number greater than 1000 copies/cell is characterized by high expression of genes E6, E7, E1, E2, E5 and E4. Expression of L1 and L2 allows the production of capsid protein, resulting in the release of the virus by programmed cell death[3].

In benign lesions caused by HPV, the virus can remain in the episomal form - free inside the cell - where after its life cycle and epithelial maturation, it is released through lysis of the cell. However, viral DNA can integrate into the genome of the host and initiate the carcinogenic process[12]. During the integration of the viral genome into the human genome, there is partial loss (E2 and L2) or total (E4 and E5) of some viral proteins[13]. In this case, the absence of E2 protein expression can lead to an increased transcription of the E6 and E7 oncogenes of HPV types such as HPV16[14].

CLINICAL ASPECTS

Characteristics lesions caused by the infection with HPV should be observed under visual inspection of characteristic clinical signs and presence of viral DNA through molecular tests. In the acute phase, the examination involves looking for the presence of condyloma acuminata (warts) and biopsy for histopathology. The presence of condyloma lesions in uterine cervix is determined by colposcopy or VIA (visual inspection with acetic acid), used to exacerbate pre-existing lesions and make it easier to detect the aceto-white epithelium suggestive of HPV infection[15]. In sub-clinical cases, colposcopy and microscopy of vaginal smears are used to search for cells with changes caused by the virus[16]. Procedures that examine the anus and rectum, as digital rectal examination, proctoscopy and anuscopy are considered effective in detecting these tumors[17].

In the latent infection, without clinical sign, sensitive diagnostic techniques such as polymerase chain reaction (PCR) should be used. Other methods can be used for

diagnosis, such as in situ molecular hybridization and hybrid capture[18]. A recent study showed that 78% of samples with dyskaryotic cervical cytology that initially tested negative for HPV could be considered positive after re-analysis using microarray tests, which show greater sensitivity than other methods[19]. HPV has been identified not only in gynecological carcinomas but also in tumors of other organs, especially of the oropharynx and upper aero-digestive tract.

Once the lesions are found, the objective of treatment involves the reduction, removal or its destruction. Recurrence of infections caused by HPV can result from latency of the virus, intrinsic failure of the therapeutic method used, new contamination or self-contamination[18]. The methods available for the clinical treatment of HPV are divided into application of topical agents, surgery and immunotherapy (**Table 1**). The choice of therapeutic treatment should be made according to patient characteristics, such as number, size and type of lesions (acuminate, flat or both) and infection site. However, it is interesting that lesions with low-risk HPV types has no clinical application, thus, cannot be recommended treatment[20], suggesting the spontaneous resolution.

INFECTION SITES

At the end of the 1960s, cervical cancer was incorrectly associated with herpes virus type II[21], and this link was adjusted in the following decade[11]. At the start of the 1970s, the association between cervical cancer and HPV was confirmed. A German research virologist, Harold zur Hausen, conducted studies in this area for years which contributed to this important discovery, and in 2008, he was awarded the Nobel Prize. Currently, it is known that HPV can be found in several other sites of infection, not only in the genital region, which was originally the basis of the link[22] (**Figure 1**). HPV is recognized as the causal factor of anogenital cancers (cervical, vaginal, vulvar, anal, and penile), and regions of head and neck cancers (oral cavity, pharynx, and larynx). However, for all other cancers describe here, the role of HPV is still under debate remaining unclear the relationship of this virus with some cancer sites.

Uterus

The World Health Organization (WHO) estimates that the number of global cancer deaths is expected to increase by 45% from 2007 to 2030 (from 7.9 million to 11.5 million deaths), and new cases of cancer in the same period are predicted to jump from 11.3 million in 2007 to 15.5 million in 2030. According to the Pan American Health Organization (PAHO), cervical cancer is projected to affect around 750,000 women by 2020 and 1 million new cases by 2050. Studies over the past 30 years show that the risk of HPV infection in the uterine cervix is very influenced by certain predisposing factors, which may influence vaccination[23]. Sexual activity, number of sexual partners, age at onset of sexual activity and encounter with sexual partners, hormone replacement therapy[24], smoking, multiparity and other sexually transmitted diseases may increase the chances of viral infection[25]. A genetic predisposition, frequency of re-infection, co-infection with more than one HPV type and altered hormone levels are also mentioned as facilitating factors for infection[26]. Individual genetic characteristics, such as the polymorphism of major proteins of innate immunity, have also been linked to HPV infection, where mannose-binding lectin (MBL) shows a high association with the risk of HPV infection[27]. A mutation in amino acid residue 72 (P72R) of p53 is also cited as a predisposing factor for HPV infection[28]. An Italian study comparing women who never smoked with current smokers showed an increased risk of HPV infection of 44.2% compared with 23.5% in never smokers, and high possibility of multiple-type viral infections was observed[29]. Predisposition to viral infection has been related to lifestyle and geographic disposition, making it important to analyze the incidence data of HPV type in particular populations.

Vagina

The incidence of invasive vaginal cancer is almost 1 per 100,000 women, where it is considered a rare disease[30]. Acuminate vaginal lesions occur in less than 30% of women with vulvar condylomatosis[18]. HPV16, 18, 30, 58, 66, and 74 are associated with cancer of the vagina[31], as well as other risk factors such as exposure to diethylstilbestrol in the uterus, immunosuppressive therapy, smoking[29], chronic irritation from the use of a vaginal pessary, radiotherapy for cervical cancer and genital herpes infections[18]. A vaginal infection is predominantly subclinical, and therefore

under-diagnosed. It is characterized by an aceto-white epithelium and condylomatosis as white dots[32]. The symptoms are thick white discharge adhering to the vaginal wall, because of epithelial desquamation, and complaints of itching, burning and dyspareunia[33].

Vulva

Vulvar carcinoma is found in 3-5% of all gynecological malignancies, and 1% of all cancers in women, with an incidence rate of 1 to 2 per 100,000 patients[34]. Squamous cell carcinomas (SCC) are the most common histological subtype, where they are observed in 90% of vulval cancer cases. It is a non-estrogen-dependent lesion[24]. HPV16 and 18 have been associated with non-keratinizing SCC in young women[35]. HPV16 is the most frequent in vulvar cancer and in the region between the clitoris and urethra[36]. Age of menarche, menopause, early sexual activity, number of partners, education, smoking, hysterectomy, and use of oral contraception or hormone therapy are well-known risk factors for vulvar carcinoma[37]. Vulvar intraepithelial neoplasia (VIN) can be classified as bowenoid and basaloid, according to the age of women affected. Bowenoid VIN affects young patients and is characterized by multiple lesions positive for HPV16, usually in bilateral areas with no hair[18], with high capacity for natural regression. On the other hand, basaloid VIN is rare and typical of advanced age with location in areas with hair, unilaterally. The signs and symptoms in vulvar HPV infection may be varied and include the presence of warts (64%), itching (20.3%), discharge (4.6%), burning (2.7%), ulcer (2,3%), pain (1.4%), spots (1.4%), bleeding (1.0%), and dyspareunia (0.5%), which may be asymptomatic in 1.85% of cases[18]. The differential diagnosis of lesions associated with HPV in the genitalia can be complex due to the number of physiological and pathological conditions that may be similar to condyloma acuminatum, such as Buschke-Löwenstein tumor (giant condyloma), verrucous carcinoma, fibroepithelial polyps and vestibular papillomatosis[38].

Ovary

Ovarian cancer is the second most common gynecological malignancy in developed countries[39]. Epithelial ovarian cancer (EOC) accounts for more than 90% of ovarian malignancies and is classified into four main histological subtypes - serous,

endometrioid, clear-cell and mucinous carcinomas[40]. HPV was detected in approximately 90% of endocervical adenocarcinomas[41] and squamous intraepithelial lesions[42]. HPV was also identified in ovarian neoplasms, as showed for ovarian and endometrial cancer tissues examined for the presence of HPV showed amplification result for E6 gene. The amplified DNA sequences were then detected with Southern blot hybridization analysis. HPV was detected in both benign (50% ovarian, 70% endometrial) and malignant ovarian (27.2%) and endometrial (37.5%) tissue samples [43]. The identification of viral DNA in ovarian and endocervical tumors may suggest the interpretation of metastasis related to areas of the genito-urinary tract. However, some authors found no relation between ovary malignance and the presence of HPV, showing the controversial condition of the infection.

Stomach

Because of the epitheliotropic nature of HPV, a link between intra-oral squamous cell carcinoma (OSCC) and this virus has been the focus of some research[44, 45]. Food has been a new target of study as a co-factor for cancer caused by HPV. *Pteridium aquilinum*, a plant commonly known as brackens, can cause chromosomal abnormalities and hematuria in cattle, and in humans, it acts as an immunosuppressive agent, reducing the capacity of the immune system's resistance to the virus[46]. In humans, it is known that quercetin (5,7,3',4'-tetrahydroxy-flavone), a flavonoid present in *P. aquilinum*, stops keratinocytes in G1 phase of the cell cycle, increasing the cyclin-dependent kinase inhibitor, p27Kip1, which is involved the expression of oncoproteins E6 and E7 in HPV16-transformed cells, leading to the failure of the cell cycle[47]. Both men and animals have the virus in its latent form when eat the bracken, and they release their toxins allowing the virus to develop. This occurs due to the preference of the toxins by lymphocytes, causing chromosomal change and intense proliferation of growth factors and progression tumor[48]. Through cytogenetic analysis of peripheral blood of animals with intoxication due this plant, Santos et al. (1998) reported that there is a possible association between cancers caused papillomavirus (PV) and the toxins originated from *Pteridium*[49].

Anus, Colon and Rectum

The association of HPV infections and epithelial abnormalities in the anus and rectum has been investigated since the 1980s[50, 51]. Dysplasias and malignancies of the colon and rectum may also be related to HPV infections, up to 74%[52]. In women, the direct relationship between cervical and anal infections is not clear. However, there is a higher prevalence of HPV in the anal canal of women with cervical intraepithelial neoplasias grade III[53]. Moreover, there is a strong correlation between anal HPV infection and HIV-positive patients of both sexes, with anal infection being associated with multiple HPV genotypes[54,55]. Family history of cancer of the colon and rectum, genetic predisposition to the development of chronic diseases of the intestine, diet high in animal fat, low consumption of fiber, and excessive alcohol consumption and smoking are described as the main risk factors associated with this neoplasm[56]. Anal cancer has a great potential for cure if detected at an early stage, but its symptoms are similar to those of adenocarcinoma of the rectum (rectal bleeding, abdominal pain, change in bowel habits and weight loss[57].

Bladder

A variety of infectious conditions, including urinary tract infection, gonorrhea, syphilis, other bacteria and viruses such as HPV, HIV, HSV, and BK have been studied as potential risk factors for bladder carcinoma[58]. However, there are discrepancies in the literature about the involvement of HPV in the etiology of cancer of the bladder[59, 60]. HPV may have a great role in the progression of specific bladder malignancies, such as transitional cell carcinoma, promoting higher stages and/or grades through the inactivation of tumor suppressors or other unknown mechanisms[61].

Penis and prostate

Penile carcinoma is a rare and potentially mutilating disease with a varied etiology, and HPV is considered a possible etiologic agent for cancer development. According to the method used for identification and geographic location, the prevalence of HPV DNA in carcinoma of the penis varies between 20 and 80%[62, 63]. One study showed that about half of the penile tumors were associated with HPV 16/18 with little presence of other genotypes[64]. According to its location on the penis, HPV can be found

in the internal prepuce (60-90% of cases), body (8-55% of cases), the glans (1-20% of cases) and the scrotum (5-20%). Viral DNA has never been found in pseudoepitheliomatous keratotic and micaceous balanitis (PKMB), but a recent study suggested that HPV infection could allow the transformation of verrucous carcinoma in PKMB[65]. The importance of HPV detection is related to the fact that men could act as a viral reservoir, transmitting the virus to their sexual partners, since it has been identified in the prostate and semen[66]. Additionally, normal tissue and prostate cancer tissue are susceptible to infection by HPV, which could be considered a risk factor for the progression of cancer[67]. HPV16[68] and 18[69] have been detected in patients with prostate cancer. The actual rates of HPV in prostatic adenocarcinoma oscillate between 0 and 75%, and many authors believe that the prostate is an important reservoir for HPV in men[70].

Head and neck

The term "head and neck cancer" includes lesions that affect regions such as the mouth and oral cavity, nose and paranasal sinuses, pharynx and larynx[71]. Thus, there are a variety of malignant and benign lesions of the head and neck that are related to HPV[72]. The mechanism of viral transmission of this type of cancer is poorly understood. It is believed that the epithelium of the deep tonsillar crypts, which is in straight contact with the lymphoid tissue, could be more vulnerable to HPV infection or transformation. Haddad et al.[73] proposed viral transmission among married couples as PCR diagnosis revealed identical viral genomes between them. Humoral response such as immunoglobulin production was also related to the viral infection[74]. In this study the authors propose that women with low levels of total secretory IgA is more susceptible to HPV infection of the oral mucosa. Respiratory papillomatosis was described in children with benign lesion with high probability of recurrence, which can be effectively treated with locally administered of cidofovir[75]. Squamous cell carcinoma of the oral mucosa is highly associated with high risk HPV[76], especially HPV26[77]. However, in HIV-positive patients, healthy mucosae may show the presence oncogenic types of HPV[78]. The patient's age, HPV type and numbers of surgeries are prognostic factors in the course of the disease, as well as the density of CD83+ dendritic cells in cancerous laryngeal tissue[79]. In the pharynx, the lesions caused by HPV, have been

described as benign. However, squamous cell carcinoma of the oropharynx was recently described as positive for HPV16 and 33, some with long-term survival after chemo-radiation treatment[80,81] .

Ear

Chronic ear infections can be attributed to bacteria, but also have been associated with the presence of HPV. Patients with cholesteatoma of the middle ear have been shown viral DNA in the ear[82]. Findings demonstrated that HPV6 and 11 were reported to be present in those types of the ear lesions[83]. This association could be related to the fact that HPV could find the possibility of replication in cholesteatoma of middle ear, since retraction pockets of epithelium and junction lines between squamous epithelium and mucosa occur[84].

Lung

Cancer of the lung is the most common type of cancer in the world. Risk factors related to this type of cancer is exposure to asbestos, the radioactive gas radon, environmental pollution, and recurrent pulmonary infections related to excess and deficiency of vitamin A. HPV6 and HPV11 are largely related to the occurrence of recurrent respiratory papillomatosis - lesions that occur mainly in the larynx – with geographic variation in incidence rates worldwide[85, 86]. The incidence of HPV in lung cancer in reviewed article varies from 0% (France-Toulouse/Cannes, Germany-Berlin + Heidelberg, Greece-Athens, USA-Texas, Japan-Kagawa) up to 79% (Greece-Athens 69%, Taiwan-Taichung 78.5%, Japan-Okinawa 79%), mainly in frozen and fixed-formalin paraffin embedded samples. In Europe and USA, the average reported incidences around 17% and 15%, respectively; while Asian lung cancer samples showed 35.7% of HPV infection rate [87].

Eyes

Infection with epidermodysplasia verruciformis-type human papilloma virus (EV-HPV) was described as capable of causing ocular cancer, with 86% of malignancies in biopsy samples of conjunctival cancer[88]. Despite the retinoblastoma are predominantly of hereditary cause, the viral DNA was found in 82.3% paraffin-

embedded samples of retinoblastoma tissue from Mexican patients with high prevalence of HPV06 (95.2%)[89]. Indian patients with retinoblastoma showed 47% prevalence of HPV16 in 57% of samples with high degradation level of pRB[90]. These observations suggest a role of HPV in the progression of this disease and lead some authors to believe that HPV can act as a co-factor in the progression of retinoblastomas[91]. A recent study described HPV05 and HPV08 prevalence in squamous cell carcinoma of conjunctiva from HIV-positive patients[92].

Breast

The etiology of breast cancer is not yet clarified, but it is known that some factors are associated with its pathogenesis, such as family history, hormones, smoking, alcohol consumption, and some viruses[93]. The possibility of involvement of HPV in breast cancer was described by Di Lonardo and collaborators[94], who detected HPV16 in 29.4% of women with breast cancer. Considering that breast region has a different response to steroid hormones, HPV infection can be greatly affected, since steroids can interact with viral LCR increasing transcription and transformation in infected cells[95]. Paraffin embedded breast carcinoma specimens screened for HPV16 and 18 showed a 24.75% incidence in breast carcinomas, suggesting that their presence in the breast could be related to development of the malignant phenotype[96]. The prevalence of alpha mucosal HPV was verified in 8% of colostrum and milk samples. Beta HPV types were highly prevalent in the epidermis of the nipple[97]. However, the findings do not support the notion that HPV plays an important role in breast cancer[98].

Skin

About 10% of the global population has some form of wart at some moment in their life, which explains its frequent finding in both pediatric clinics and adult lesions[18]. Normally, occurs regression, but warts may progress into malignant skin cancer during immunosuppression, where the new type of HPV 117 had been found in cutaneous warts[99]. The transmission of HPV seen in clinical dermatology can occur via sexual activity (anogenital warts and orally), skin contact (contaminated objects and surfaces) or perianal contact (uterus). The association of HPV with exposure to ultraviolet light[100] has been suggested to be of relevance for the development of non-melanoma for up-

regulated the transcription from HPV-8, 93 and 96 [101] and a risk factor for the formation of epidermodysplasia verruciforme (EV), where HPV5 and 8 were more associated[102].

Blood

Circulating HPV DNA has been detected in advanced stages of cervical cancer, where studies have shown that the prevalence of HPV16 in plasma could be an important prognostic marker of advancing cervical disease[103]. HPV16 was also found in lymph nodes of patients with early stage cervical carcinoma, where it was localized in mononuclear cells of 92.3% of patients with cervical infections[104]. These data suggest that these cells could carry the virus through the blood. Hypothetically, HPV in plasma could be the result of bloodborne virus particles or necrosis of cells resulting in release of viral DNA into the circulation[105]. Viral detection in the blood of patients with cervical carcinoma with HPV was 11.8% whereas papillomavirus DNA in the lymph node was detected and correlated with metastasis[106]. Since HPV is an epitheliotropic virus, its presence in the bloodstream, associated with peripheral blood mononuclear cells can be interpreted as a type of metastasis[107, 108].

Others sites

The hands can act as a medium in many diseases, where fingers and nails are pointed out as an alternative mode of viral transmission, in addition to the contact with the mucous membranes. In 27% of patients, an association was found between HPV type detected in hands and genital lesion of the same patient[109]. Periungual squamous cell carcinoma (SCC) is frequently associated with alpha-HPV infections, suggesting that capacity plays an important role in tumorigenesis, noting that beta-HPVs are rare[110]. The wart lesions caused by HPV can also lead to nail dystrophy when infection occurs in the matrix. Genotyping has shown the presence of HPV16, 34, 35 and 57 with SCC of the nail[111]. HPV15 was also associated with an X-linked dominant disorder (incontinentia pigmenti) with subungual tumors[112].

The presence of HPV in hairs plucked from the pubic and perianal regions of patients with genital warts was investigated, since they are of clinical importance in the development of anogenital warts. HPV6 and 11 was found in 24% of hair samples from the pubic area and 36% from the perianal region[113]. Viral presence and distribution in

the plucked eyebrow hairs showed different HPV genotypes: SIBX4, SIBX5 and SIBX6[114]**.** These results suggest that hair follicles can act as an endogenous reservoir for HPV, which may play a role in recurrences of genital warts.

FINDINGS OF HPV WITHOUT CYTOLOGY ALTERATION

Shukla et al. (2009) showed in their study the prevalence of HPV infection in cancers of different organ sites in Indian population[115]. Despite the large number of different types of cancer in which HPV was found, these numbers are low in people infected by this virus. In women infected by HPV, 90% of the infections resolve spontaneously, only 10% can become persistent which 3-4% can progress to intraepithelial lesions[116]. The incidence of high-grade lesion will achieve 0.7 to 1% and 0.1% may progress to invasive cancer if not detected and treated early[116]. Patients initially diagnosed as positive for HPV and subsequently described with undetectable levels of HPV showed higher rates of CIN regression (63%) than patients with HPV persistence (41%)[117].

Some controversial findings of viral DNA in putative uninfected samples for HPV or in control groups[118,119] leads to a reflection about the viral history. HPV colonization has been suggested as protective to the host and this virus could exist as commensal without causing any detectable existence or can be lead to a verrucous structure[5]. Similar association of protective effect has been shown for Epstein–Barr virus, human cytomegalovirus and herpes virus, being able to confer resistance to infection by bacterial pathogens[120]. HPV was also indicated to be an opportunistic virus[121] once the pathogenicity is activated for co-infections with the HIV[122].

Moreover, there is a tendency to abolish the HPV type classification in high and low risk, whereas HPV6 has been isolated in cancer penile under single and multiple infections[123]. Therefore, it seem that in the future HPV risk type will be related to the characteristics of the host through biochemical and immune imbalance associated with risk factors for HPV acquiring that are responsible for the progression of precursor lesions to cancer.

FINAL REMARKS

Infection of the cervix is the most studied clinical occurrence involving HPV, but this virus can be found in several sites in the human body. Only now, with more powerful technologies such as high sensitive HPV chips, the detection of HPV at different body sites have been described, usually involving virus types other than cervical ones. Reviews and papers in this area make us wonder whether HPV can occur in at a certain site in humans without causing disease appoint the possibility that HPV acts as an opportunist microorganism. This fact can explain the findings of this virus in the body without resulting in clinical signals and in predisposing moments like immune depletion developed the pathogenicity. Further studies should clarify whether the presence of the virus in different organs can be considered a rare clinical finding or simply something that has been overlooked, and therefore under-diagnosed.

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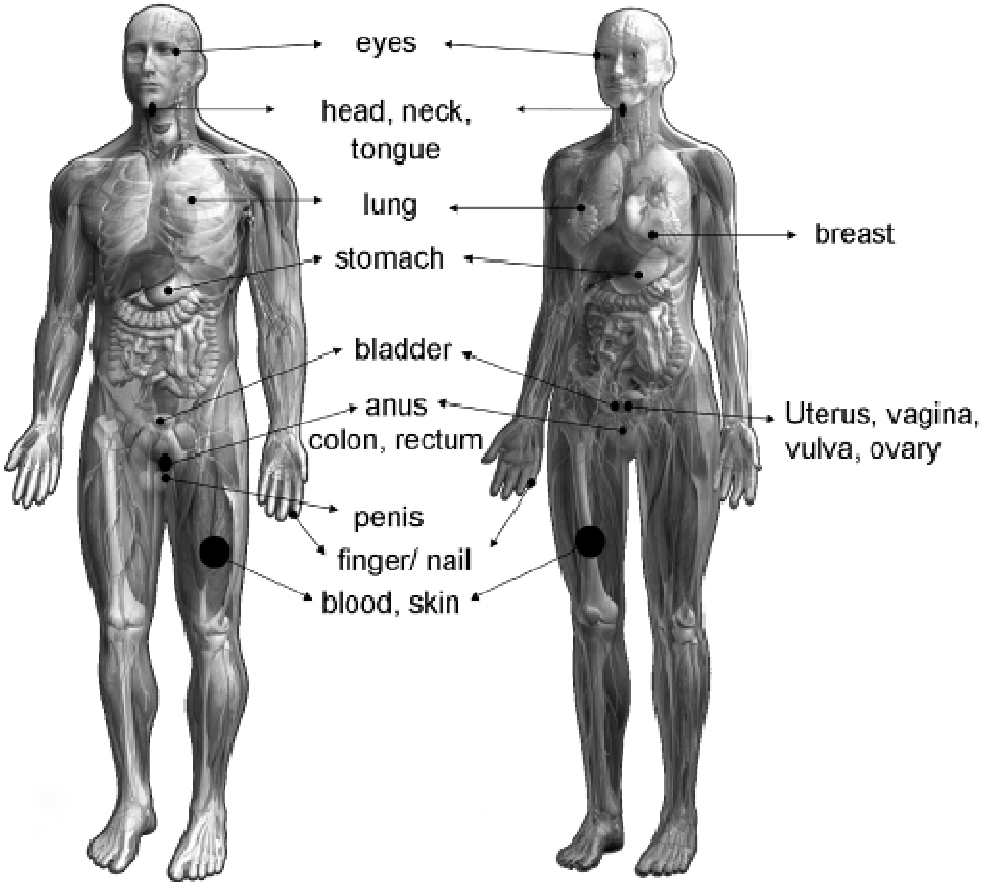

Figure 1. Schematic representation of possible infection sites of human papillomavirus. Modified from Zygote Media Group, Inc.

Table 1. List of treatment according to the clinical lesion caused by Human papillomavirus. [9, 15]

	TREATMENT	APPLICATION
Topical agents	Trichloroacetic acid (80-90%)	Lesions vulvar and penile.
	5-Fluorouracil (5%)	Lesions on the penis, male urethra, vulva and vagina.
	Interleukin-2	Refractory lesions caused by HPV.
	Podophyllin (25%)	Skin lesions
	Interferon	After surgical removal of visible lesions, as adjuvant treatment.
	Imiquimod (5%)	Vegetative lesions.
Surgical treatments	Curettage	Verruciform lesions.
	Conization and excision to high frequency	Intraepithelial neoplasia located in the area of transformation.
	Excision with scissors	Verruciform lesions, especially exophilic and pedunculated.
	Excision with scalpel	Intraepithelial neoplasia of the skin and mucosa.
	CO_2 laser	Lesions on the vulva, vagina, uterine cervix and urethral.
Homeopathy	*Thuya occidentalis*	Vegetative lesions such as warts, papillomas, condylomas and excrescences of various types.
	Acupunture	Adjuvant treatment of papillomatosis lesions.
Immunotherapy	Retinoid	Viral lesions of the skin and mucosa and help in the surgical treatment after invasive cancers, precancerous skin lesions, cervix and vulva.
	Isoprinosine	Relapse after treatment with physical agents in condylomatous lesions, lesions associated with intraepithelial neoplasia, and lesions that are refractory, recurrent and extensive.

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6. CAPÍTULO II

1. TÍTULO: Comparative efficiency of bacterial systems for heterologous beta-galactosidase expression (Note)

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Rapid method for visual identification of recombinant bacteria expressing β-galactosidase in liquid media

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Keywords: *E. coli*, colorimetric assay, beta-galactosidase, X-gal

A B S T R A C T

In this study we successfully developed an easy method to detect the expression of heterologous β-galactosidase in *E. coli* strains during bacterial growth. The colorimetric assay was used together with SDS-PAGE to determine the higher efficiency of ORIGAMI(DE3) to express b-galactosidase gene with low background of bacterial proteins.

Recombinant protein expression systems based in *Escherichia coli* are commonly used due to its easy handling, well-established protocols and high protein yield and purity, associated with the low cost of cultivation (1). Different alternatives have been used for increased activity in this system, like codon usage, adjustment of cultivation parameters and selection of expression strains (2). The commonest β-galactosidase assay is based on hydrolyses of the synthetic compound o-nitrophenyl-β-D-galactoside (ONPG) that is also recognized as chromogenic substrate (3). The aim of this study was to develop a method to observe the expression of cloned β-galactosidase in bacterial strains during the growth and select the most adequate recombinant according to the protein background profile.

We used *E. coli* BL21(DE3) containing plasmid pET16b/lacZ (Novagen®) for recombinant expression of β-galactosidase. ORIGAMI(DE3) (Novagen®) and KRX (Promega®) were used for comparison after cloning pET16b/lacZ. pET16b empty was used as control for cloning and expression steps. The plasmids were transformed in *E. coli* competent cells by heat shock (4) and incubated overnight in plates with Luria-Bertani (LB - tryptone 0.5%, yeast extract, 1.0% NaCl) media containing antibiotic at 37°C. Recombinant colonies were selected by ampicilin-resistance and identified by plasmid extraction. Strains of *E. coli* were grown overnight at 37°C in Erlenmeyer flasks containing 10mL LB medium with 100 $\mu g.ml^{-1}$ ampicillin under 150 rpm in orbital shaker. Cells were re-inoculated in 250mL flasks with 50mL of LB medium growing at 37°C and 150 rpm until OD_{600} 0.4 to 0.5. Then, the flasks were transferred to 25°C condition and induction of the LAC promoter was performed with addition of 0.5 mM IPTG (isopropyl β-D-1-tiogalactopiranoside). Promoter induction was observed by visual inspection during the first six hours after addiction of 80$\mu g.ml^{-1}$ ONPG or 5$\mu g.ml^{-1}$ X-Gal in the culture, under light protection. After 16 hours of induction, the cells were centrifuged at 3,000 x *g* for 30 min and the pellet suspended in 4mL of breaking buffer (50 mM sodium phosphate pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol). Glass beads were added for corresponding pellet volume for mechanical

lyses with four cycles of 30 seconds ice-vortex. Supernatant was harvested at 3,000 x *g* for 20 minutes at 4ºC and analyzed in SDS-PAGE 15%.

A colorimetric screening method was analyzed with *E. coli* strains cloned with LacZ gene. Two substrates (ONPG and X-gal) were added to the media during bacterial growth. All strains were able to express the recombinant β-galactosidase and BL21(DE3)/pET16b/lacZ culture is showed in **Fig.1**. Yellowish color was observed in the first two hours of growth induction, indicating β-galactosidase activity once it hydrolyses ONPG, analogous lactose substrate. The blue color was obtained with X-gal (5-bromo-4chloro-3-indolyl-β-D-galactoside) chromogenic substrate in the same time, as response of enzyme activity. An advantage of this method is the use of X-gal in substitution of ONPG for β-galactosidase assay, saving money and timing for beta-galactosidase screening directly in growth culture. This methodology is easy to realize in initial cloning steps and does not require special equipment or personal training as observed for conventional method, like ONPG color reaction and histochemical staining (5). Besides, the insolubility and stability observed for X-gal allows the storage of the sample for further studies, maintaining the color (6). This method is not indicated for recombinant systems that produce low concentration of the target enzyme, once colorimetric assays for β-gal have detection limit around 100pg compared to chemiluminescent assay that detects fentogram level (7).

Bacterial protein extracted was analyzed in electrophoresis after the induction, in order to determine the most adequate *E. coli* strain for β-galactosidase production. It was possible to visualize the correspondent band around 116KDa, for all strains. ORIGAMI(DE3), that carries mutations in thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, showed lower background protein (**Fig. 2**) without losing the concentration of β-galactosidase. BL21(DE3) showed similar band profile for β-galactosidase, but the homologous proteins were not inhibited by the presence of the inductor IPTG that leads to lower final concentration of the target protein. Its genotype *lon* and *ompT* protease deficient could allow the maintenance of bacterial proteins

in the medium, reducing the efficient of the purification step. Despite ORIGAMI(DE3) has no protease deficient genotype, its advantage in disulfide bond formation for proper folding could lead to an increased stability of β-galactosidase protein in the media. BL21(DE3) was previously described for production of recombinant human protein fragments showing lower yield than Rosetta(DE3) strain (8). KRX showed the similar profile of BL21(DE3) with higher protein background.

In conclusion, the use of X-gal showed to be a fast method (around few hours comparing with tradicional protocols of days) to screen strains that produce β-galactosidase following the expression visually during bacterial growth. This method could also be applied for cloning systems that use β-galactosidase report gene and can be scaled down for the researcher convenience. Additionally, ORIGAMI(DE3) strain was the most indicated system for heterologous expression with higher possibility of enzyme recovery from the media due to the lower concentration profile of homologous protein.

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Figure 1. Colorimetric assay performed with *E. coli* BL21/pET16b-lacZ grown in LB medium supplemented with ampicillin and IPTG: (A) control without substrate, (B) ONPG and (C) X-Gal.

Figure 2. Polyacrylamide gel containing protein extracted of *E. coli* strains induced with IPTG. Lane (2) KRX wild-type, (3) KRX/pET16b/lacZ, (4) KRX/pET16b, (5) ORIGAMI(DE3) wild-type, (6) ORIGAMI(DE3)/pET16b/lacZ, (7) ORIGAMI(DE3)/pET16b, (9) BL21(DE3)/pET16b/lacZ. Lanes (1) and (8) protein molecular weight marker (GE). Arrows show the band of β-galactosidase.

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Figure 1

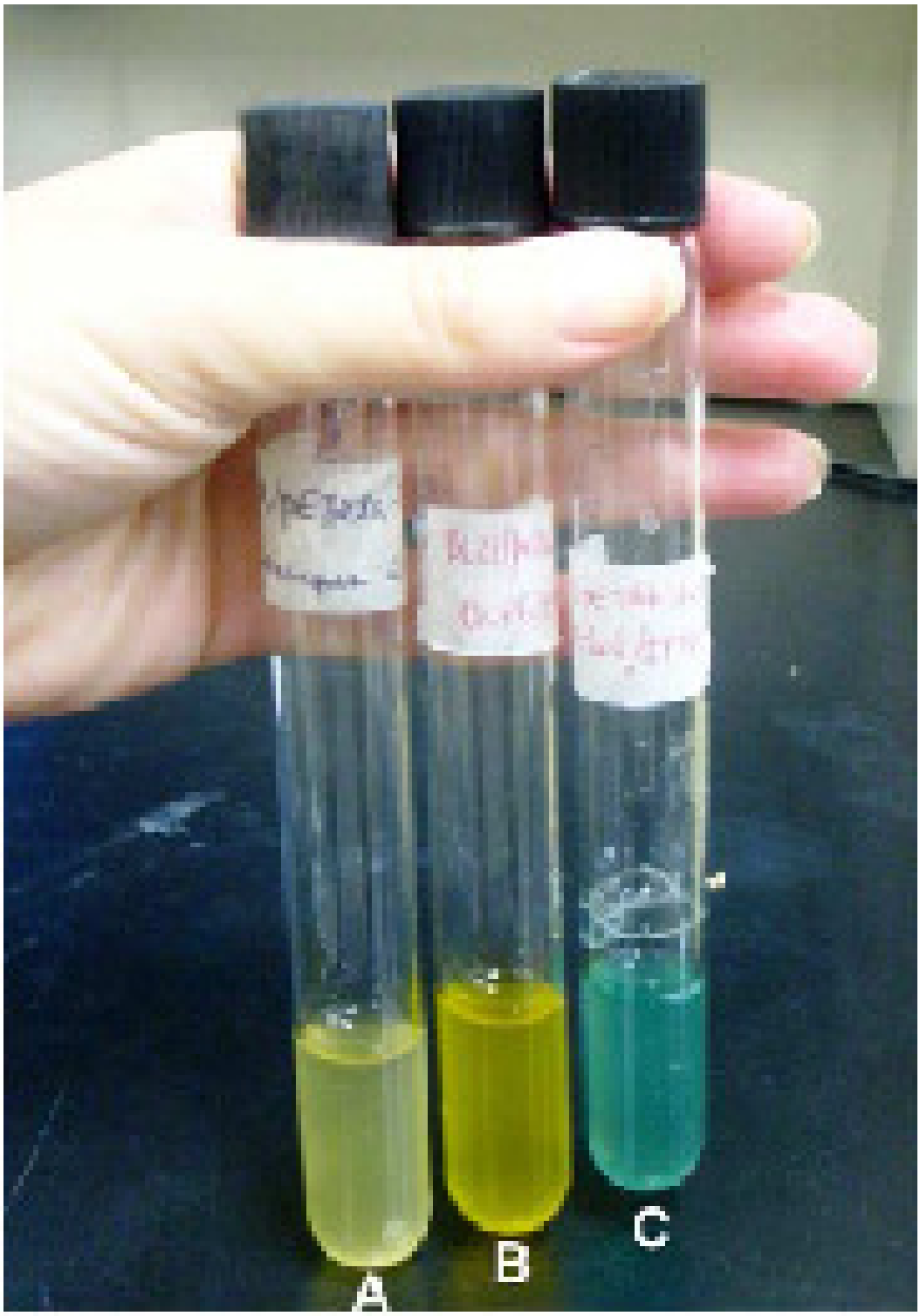
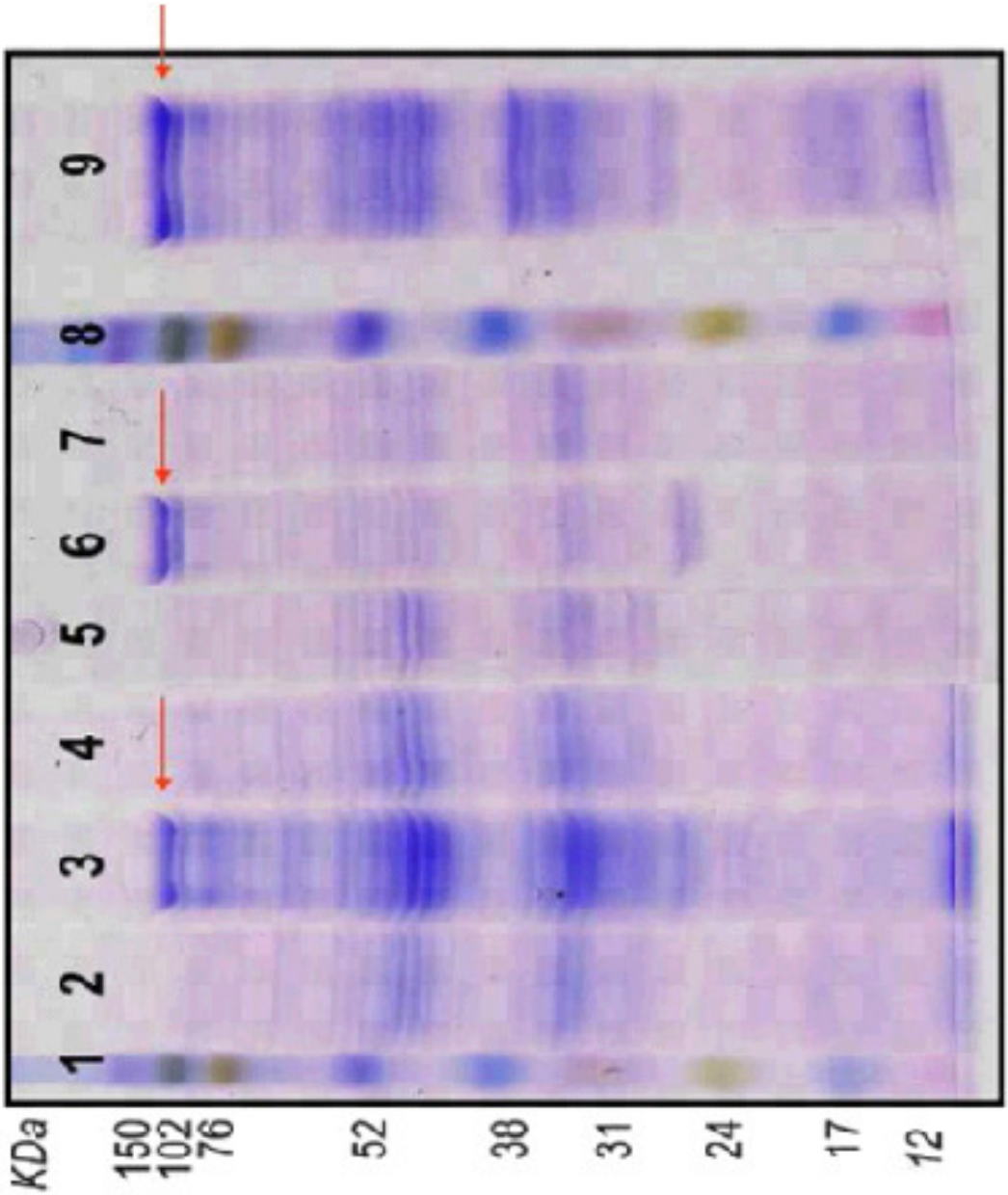


Figure 2



7. CAPÍTULO III

1. TÍTULO: Detection, genotyping and variant analyses of human papillomavirus 16 isolates from Northeast Brazilian population by E6 and E7 ORF analysis.

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3. REVISTA: Virus genes (Impact Factor: 1.705)

Detection, genotyping and variant analyses of human papillomavirus 16 isolates from Northeast Brazilian population by E6 and E7 ORF analysis

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SUMMARY

Several studies show that molecular variants of human papillomavirus type 16 (HPV16) have different geographic dissemination. E6 and E7 oncoprotein acts in cell transformation. E6, E7 and L1 genes have been used as target for researching HPV types variant. In this study, we examined the sequence of E6 and E7 genes from HPV16 found in cervical specimens collected in Northern Brazil. Analysis of E7 ORF sequence evidenced the majority samples as European variant and only four patients related to have Asian-American HPV16 variant. However, the analysis of E6 ORF in these Asian-American samples showed the absent of G351A mutation, suggesting a putative new variant. This study is potentially relevant to understand the virus adaptation to the host and its evolutionary route in human infection.

Keywords: Human papillomavirus; HPV16 variant; E6 ORF; E7 ORF; cervical samples.

INTRODUCTION

Papillomaviruses (PVs) are currently represented by more than 200 genotypes (Bernard, 2005). They are divided into 16 PVs genera (Bernard et al., 2010), and alpha-papillomavirus is considered the most clinically important genus as it includes the largest number of Human Papillomavirus (HPV) that cause lesions, including cervical neoplasia. HPV types can be largely grouped according to the degree of cell damage that they are able to cause, resulting in high or low risk (Stanley, 2001). Analysis of HPV types is important for several reasons, among them the fact that HPV types are tissue specific and generally produce different types of lesions, in particular those that are either benign or malignant (Lizano et al. 2009). In malign lesions caused by HPV, viral DNA can integrate into the genome host and initiate the carcinogenic process (Peitsaro et al. 2002). HPV genotype 16 (HPV16) is the most frequent high risk papillomavirus found in lesions of genital tract. The expression of oncoprotein E6 and E7 in HPV16-transformed cells was found to lead to the failure of cell cycle (Doorbar, 2006; Beniston et al. 2003). De Villiers et al. (2004) and Bernard et al. (2010) described the topology of phylogenetic trees, quantitative thresholds in nucleotide sequence comparisons and biologically distinguishing features (host species, target tissues, pathogenicity, and genome organization) that determine the classification of PVs on the level of genera. These virus types have co-evolved with their hosts and showed higher genotypic alteration today (Lizano et al. 2009). HPV genome is defined as a new type when the distance or dissimilarity of nucleotide sequence is higher than 10% compared with other known HPV types in L1 open reading frames (ORFs) (De Villiers et al. 2004). It is also possible to use E6 and E7 ORFs, combined with L1, since these genes have showed as conserved regions in genome (Chan, 1995). HPV from the same genotype are referred as variants when differs in 2% ORFs and 5% in untranslated region (UTRs) (Bernard et al., 1994). Nowadays, other regions not well understood were used to study genotype variation, as observed for LCR, E2 and E5 ORFs (Kocjan et al. 2009). Evidence from databank sequences suggests the existence of at least eight HPV16 variants. Analysis of HPV16 variants has several objectives, once sequence variations can be used as markers for monitoring HPVs in defined populations. In this way is important to establish databases for development of efficient diagnostic tools and vaccines and application to epidemiological studies (Cai et al. 2010; Stewart et al., 1996). Studies with HPV intra-type variations may to elucidate differential abilities to

activate cell-signaling pathways, involved in cell survival and proliferation (Contreras-Paredes et al. 2009) and evolutionary and taxonomic studies (Bernard et al., 2010). In this study, we used cervical specimens for HPV genotyping identification by Papillocheck® and sequenced E6 and E7 ORFs to search for SNPs and identify HPV16 variants in Northeast of Brazil.

RESULTS AND DISCUSSION

A total of 133 samples were analyzed for HPV infection and 54.9% (73/133) were positive for HPV detected by Papillocheck® (Greiner Bio-One). All HPV types covered by Papillocheck were found, except for HPV45. It was observed that 11 patients had multiple infections. Twenty samples found as positive for HPV16 were submitted to variant analysis.

E6 and E7 variants in HPV16

Specific primers for E6 and E7 oncogenes identification were developed to target as flanking the target gene (**Figure 1**), and identify the HPV16 variant present in cervical samples collected from Northeast Brazil. E6 and E7 ORFs isolated from different world regions shows several amino acid substitutions in consequence of nucleotide changes (**Table 1**).

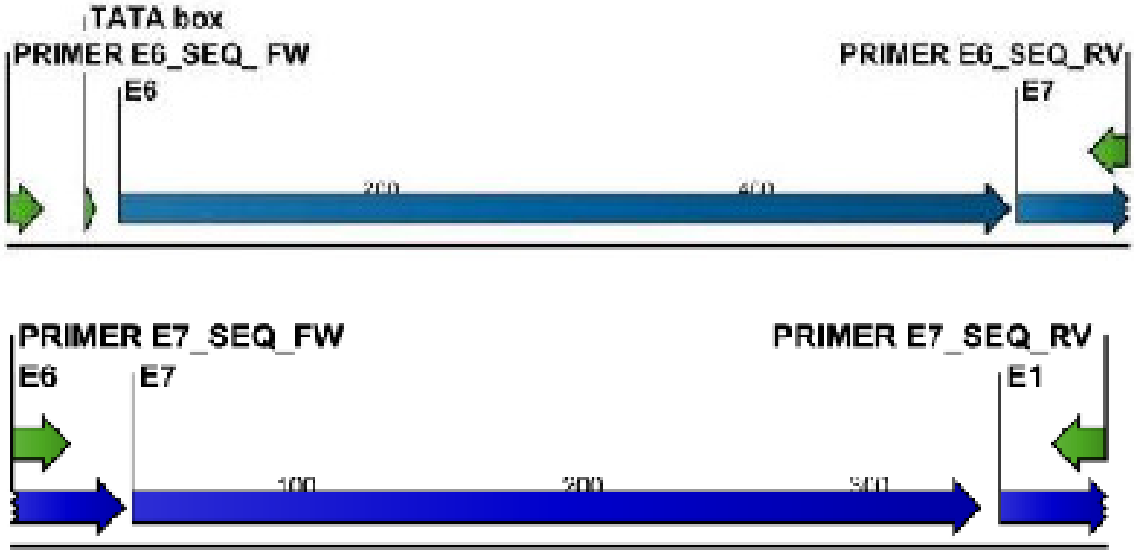

Figure 1. Binding sites of specific primers developed for sequencing E6 ORF (A) and E7 ORF (B) from human papillomavirus type 16.

Table 1. Mutation points in E6 and E7 ORFs variants from human samples infected with human papillomavirus type 16 compared with HPV16 genome model (NC_001526). Oncogenes polymorphism and oncoprotein alteration were found in population of Amazonian, African, Asian American, Chinese, East Asian and European German. HPV16 E7 ORF isolated from European German (AF536179) was identical to E7 HPV16 model, showing no mutation.

Variants associated	E6 gene mutations	E6 oncoprotein alteration	E7 gene mutations	E7 oncoprotein alteration
European German (AF536179)	A (49) G T (268) G	Arg → Gly Leu → Val	Not	Not
African type 1 var (AF472508)	A (1) C G (50) C C (61) G and G (63) T T (204) A and A (207) G C (253) T	Met → Leu Arg → Thr Gln → Asp No change His → Tyr	A (57) G T (228) C T (234) G	No change No change No change
African type 2 var (AF472509)	T (27) C G (50) T C (61) G and G (63) T T (204) A and A (207) G C (253) T A (321) G	No change Arg → Ile Gln → Asp No change His → Tyr No change	A (86) G T (228) C T (234) G	Asn → Ser No change No change
Amazonian (HM057182)	A (1) C G (50) C C (61) G and G (63) T T (204) A and A (207) G C (253) T A (365) G	Met → Leu Arg → Thr Gln → Asp No change His → Tyr Lys → Arg	T (83) C and A (84) G A (85) C, A (86) C and T (87) C G (88) T and A (89) T T (228) C T (234) G	Leu → Ser Asn → Pro Asp → Phe No change No change
Chinese (EU918764)	A (238) G C (253) T A (348) G A (360) C	Ile → Val His → Tyr No change Glu → Asp	A (85) C	Asn → His
Asian American (AF402678)	G (63) T T (204) A and A (207) G C (253) T T (268) G G (351) A A (450) G	Gln → His No change His → Tyr Leu → Val No change No change	T (171) C T (228) C T (234) G	No change No change No change
East Asian (n° AF534061)	T (96) G	Asp → Glu	A (86) G T (285) C	Asn → Ser No change

Amplicons with 382bp and 599pb were obtained for the amplification of E7 and E6 region, respectively (**Figure 2**). This material was used for sequencing procedures to observe the SNP in both ORFs.

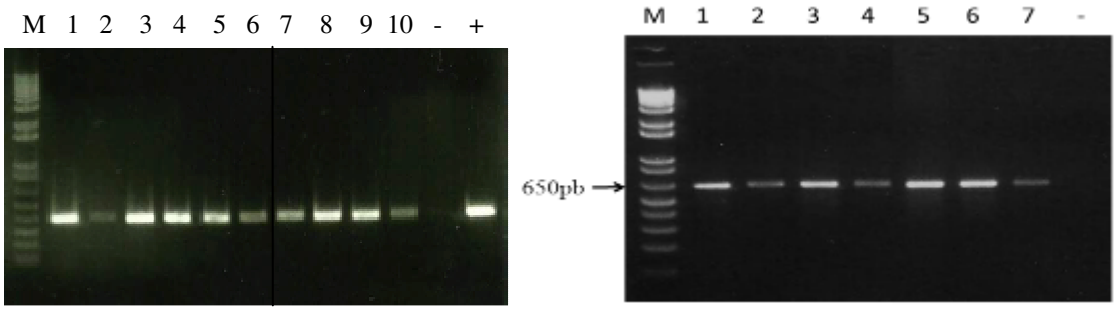

Figure 2. Amplification by PCR performed on HPV16-positive cervical lesions with specific primers. (2A) Amplification of E7 ORF in 10 specimens: Lane (M) 1 Kb Plus Ladder, (1-10) E7 amplicon, (–) and (+) were negative and positive control, respectively. (2B) Amplification of E6 ORF in 7 specimens: Lane (M) 1 Kb Plus Ladder, (1-7) amplicon of samples, (–) negative control.

The European German variant was prevalent in the samples analyzed with 75% (15/20) and four patients showed polymorphism for E6 and E7 oncogenes (**Table 2).** Three SNPs (T171C, T228C, G234T) found in E7 ORF showed association with the Asian-American variant of HPV16.

Table 2. Single Nucleotide Polymorphisms (SNPs) in HPV16 E7 and E6 ORFs observed in specimens isolated uterine cervix.

Patients	**E7 SNPs**	**Mutation**
60, 104, 186, 545	T (171) C	Silent
	T (228) C	Silent
	G (234) T	Silent
	E6 SNPs	**Mutation**
104, 186, 545	G (63) T	Gln → His
	T (204) A A (207) G	Silent
	C (253) T	His → Tyr
	T (268) G	Leu → Val
	A (450) G	Silent

This variant was also found in human leukocyte antigen (HLA) in Brazilian population, beyond European and African variants (de Araujo Souza et al., 2008). However, our results do not support previous studies that showed a high frequency of Asian-American variants in cervical neoplasia among women with multiethnic origin (Junes-Gill et al. 2008).

These samples were submitted to E6 ORF sequencing and showed similar profile of Asian-American variant, except for the absent of SNP351 (**Figure 3**). All specimens analyzed for E7 gene showed no amino acid changes, but E6 sequencing showed three amino acidic modifications: Q63H (Gln→His), H253Y (His→Tyr) and L268V (Leu→Val). These mutations in amino acidic chain reverberate in alteration in conformation of E6 oncoprotein and its interaction with human proteins.

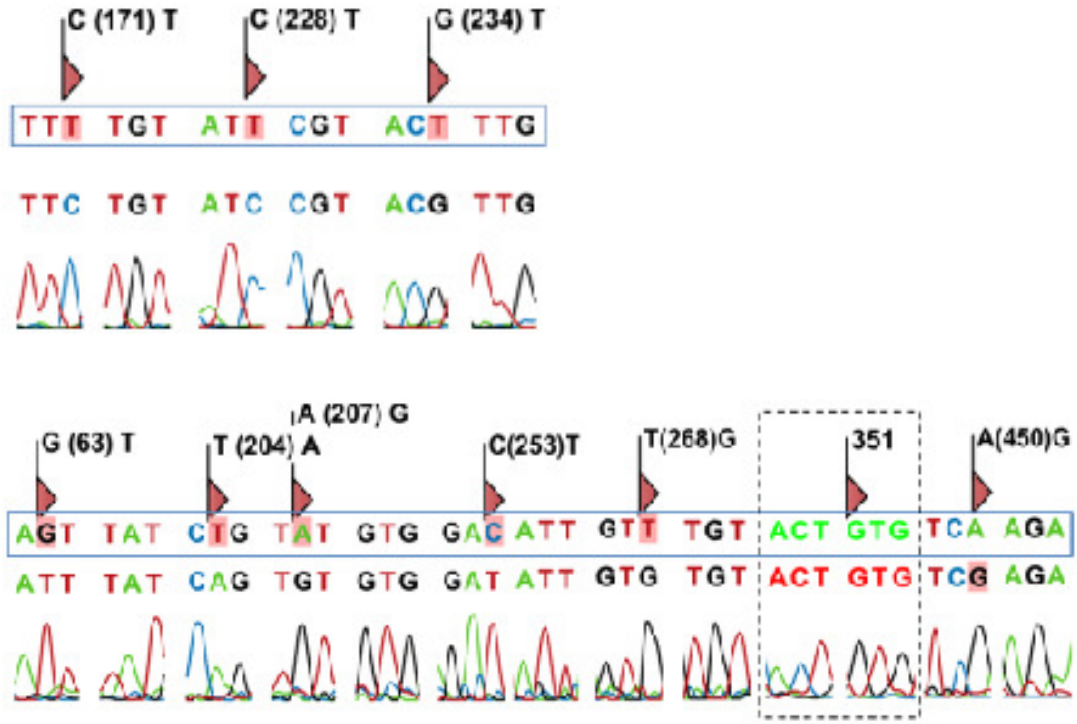

Figure 3. Electropherogram with annotated mutation found HPV16 E7 and E6 ORF. **3A.** Position 171, 228 and 234 from E7 ORF characteristic from Asian-American variant. **3B.** Position 63, 204, 207, 253, 268 and 450 showed for E6 ORF with boxed referred SNP351 absent in E6 ORF, avoiding the full characterization of Asian-American variant.

One sample showed a conflicted area in eletropherogram, suggesting the presence of both peaks corresponding to European and Asian-American profiles simultaneously (**Figure 4**). These results suggest a possible infection of both variants HPV16 in the same sample.

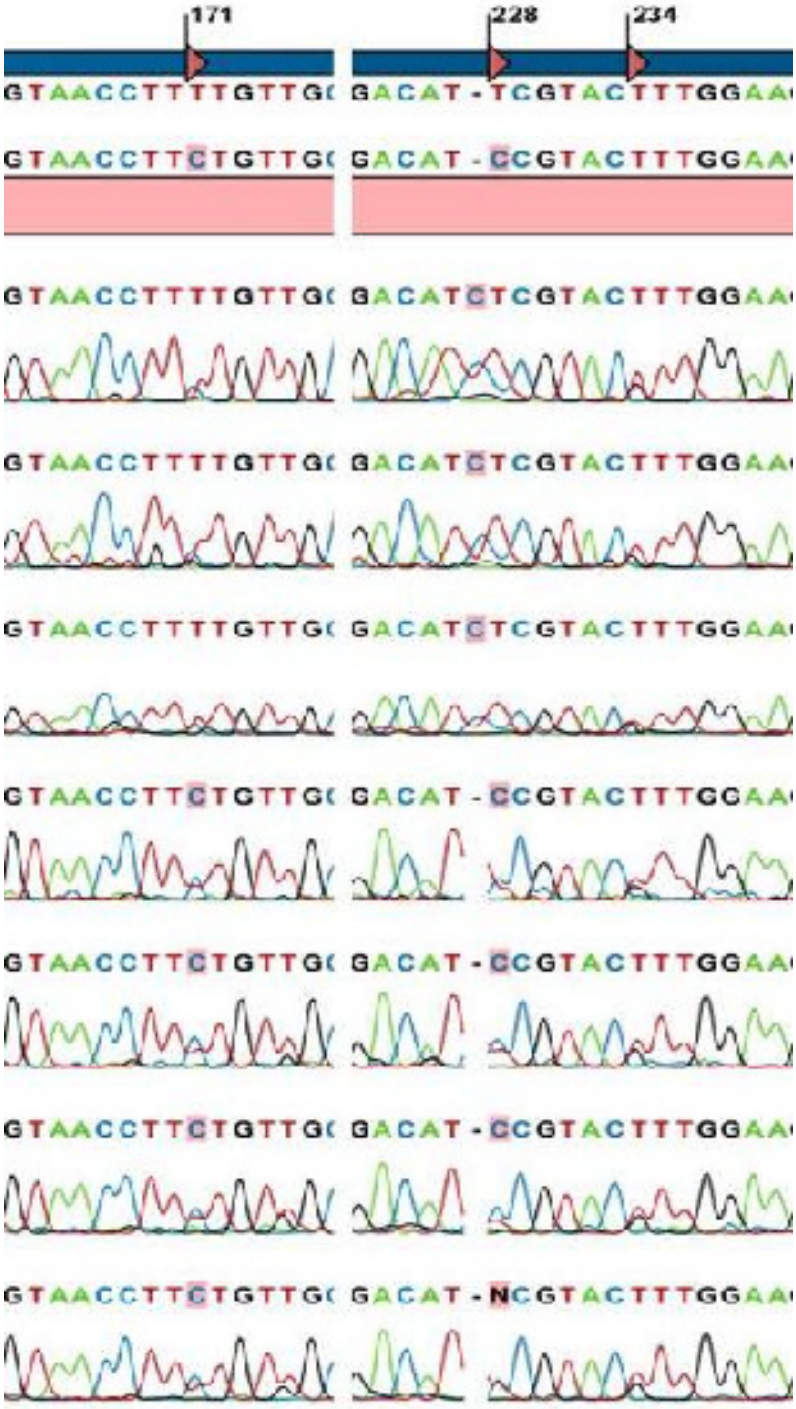

Figure 4. Electropherogram showing conflicting region that suggest a co-infection of Asian-American and European variant HPV16 based in E7 ORF analysis.

It is known that nucleotides changes can alter the interaction bind with DNA transcription factors. It happens in E2 gene from HPV16 (Graham et al. 2000), which C3684A variant in E2 DNA binding domain seems to result in significant increased frequency in high-grade lesions (Giannoudis et al. 2001).

The range of E7 gene variations found in HPV16 isolated from cervical samples from Royal Liverpool Hospital did not exceed 0.9% (3/343) (Giannoudis et al. 2001). By another hand, nucleotide sequence variations in E6, L2 and L1 ORFs showed a variation of 1.8% that allowed the suggestion of new variant in American population (Yamada et al. 1995). In this study 21% of the patients analyzed (4/19) showed alterations in E6 ORF, indicating that further experiments should be performed. Increase in patient numbers and inclusion of L1 and L2 ORFs in sequencing steps should provide more appropriated conclusions.

Prognosis for the variants

Molecular methods are useful to available the detection of HPV presence and genotype, like in situ hybridization (Oliveira et al. 1994), PCR-RFLP (Maver et al. 2010; Medina et al. 2010) and real-time PCR (Kocjan et al. 2008). However, the technique that was used (PCR-sequencing) is an assay simple and robust that can show great value for epidemiological studies. Sequence variation is important for the development of more precise diagnostics approaches and vaccines, as well as for epidemiological investigations and phylogeny studies of HPVs. A clinical and epidemiological study realized in Mexico suggested that Asian-American HPV16 variants may be more oncogenic than European variants (Berumen et al. 2001). In our study, patients with more aggressive tumors were found with a higher frequency in European variants whereas a lower frequency for Asian-American variants (**Table 3**).

Table 3. Clinical condition of the cervical sample analyzed and its relation with Human papillomavirus type 16 variant.

Cervical sample	**Incidence**	**HPV16 variant**
CA colo	11	Asian American (01)
		European German (10)
Cervicallesions	8	Asian American (02)
		European German (06)
Warts's Vaginal	2	European German (01)
		European German and Asian American (01)

Observations that E2 gene is transcribed appropriately in Asian-American HPV16 tumors, is related to the 16 mutations that does not repress the transcription of E6/E7 oncogenes (Ordonez et al. 2004). These authors suggest that HPV16 Asian-American variant starts earlier the E6/E7 expression in the cervical infection that may explain the fact that women infected by this kind of HPV can develop invasive cancer 8 to 12 years before women with HPV16 European variant (Ordonez et al. 2004).

HPV16 E7 variants were not able to result in high-added changes in carcinogenesis, due to the low variability and absence of exchange amino acidic founded. However, mutations in E6 ORF were related to amino acid change, which can influence in protein interaction. Therefore, mutations in HPV ORFs seem to control the viral action since the relation between viral oncoprotein and human proteins has great importance for cancer developing.

METHODS

Clinical samples, DNA extraction and HPV identification

The present study was conducted with 133 patients from Pernambuco (Recife and Olinda)., in the coast of Brazilian Northeast Samples were collected from endocervix, vagina and vulvar regions using citobrush cell collector and stored in saline buffer (Promega® Corporation). DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega® Corporation) and samples were analyzed by Biofotometer® (Eppendorf). Genotyping was performed by hybridization with Papillocheck®.

Molecular variant analysis

All PCR reactions were performed in 0.2 ml reaction tubes, each containing 1 µl DNA obtained from extraction, 6.25 µl of GoTaq® Hot Start Colorless Master Mix (Promega® Corporation), 10 pMol of each specific primers and water up to 12.5 µl. The cycling conditions used were 2 min at 95 °C, 30 cycles of 30 sec each at 95 °C, 54ºC and 72ºC, and 5 min at 72 °C. All PCR amplifications were carried out on the MyCycler™ Thermal Cycler (Bio-Rad). After this, the amplicon was examined on 1% agarose gel electrophoresis and visualized under UV light. Information about the primers used for amplify and sequencing of DNA is described in **Table 4**. The amplicons were purified using ExoSAP treatment (USB) and each clinical specimen was subjected to dideoxy sequencing in MegaBACE 750 (GE, Life Science), following the manufacturer instructions. The basecalling raw data was analyzed by Sequence Analyzer (GE, Life Science) and patient's contigs were assembled using the CLC Main Workbench version 5.7.1.

Table 4. Primers used to detected variation in E6 and E7 ORFs from Human papillomavirus type 16 present in clinical specimens.

Oligonucleotide	Target genome	Primer Sequence (3'- 5')
Fw E7.16_seq	520-540	TTGCAGATCATCAAGAACACG
Rv E7.16_seq	883-902	GTACCCTCTTCCCCATTGGT
Fw E6.16_seq	24-43	CTAAGGGCGTAACCGAAATC
Rv E6.16_ seq	601-622	CAGTTGTCTCTGGTTGCAAATC

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8. CAPÍTULO IV

1. TÍTULO: Cloning of E7 by Human Papillomavirus type 16 using KRX strain of *Escherichia coli* to expression
2. AUTORES: Monique Ferraz de Sá Beltrão, José Luiz de Lima Filho, Danyelly Bruneska
3. REVISTA: Journal of Biotechnology (Impact Factor: 2.881)

Cloning of E7 by Human Papillomavirus type 16 using KRX strain of *Escherichia coli* to expression

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Key words: papillomavirus, HPV-16, E7 oncoprotein, *Escherichia coli*, KRX, recombinant protein.

ABSTRACT

Cervical cancer is the second neoplasia more common in women and is mainly caused by human papillomavirus (HPV). HPV16 is a high-risk type that is responsible for 58% of all cervical cancer cases. Three oncogenes, E5, E6 and E7 are involved in malignance and maintenance of the virus inside human cell. E7 oncogene is transcribed in an early stage with the purpose of deregulating the cell cycle by pRB inactivation. The cell cycle deregulation leads to an alteration in cell morphology and physiology, and may cause cancer. In this work, we cloned E7 oncogene in *Escherichia coli* KRX strain as expression system.

Keywords: *Escherichia coli*; HPV; E7; cloning; KRX; protein expression

INTRODUCTION

Human papillomavirus (HPV) is a virus of tumor of DNA capable to transform the epithelial cells. It can be classified as high and low risk, in accordance with the potential of malignant induction to the transformation (Munoz et la 2003; Wright et al 2006). HPV is the main etiological agent involved in 99% of cervical cancer cases with HPV16 present in most genital warts and cervical cancer. Invasive cervical cancer is the second most common cancer in women worldwide (Schiffman et al, 2011), with more than 500 000 cases of invasive cervical cancer per year worldwide (Bosch et al 2002). HPV16 is responsible for the higher prevalence of cervical cancer, while HPV18 is associated to approximately one half of squamous cell carcinoma and 12% of cervical cancer (Chang et al, 2005; Altekruse et al, 2003).

Structurally, HPV genome consist of 8000 bp composed by early genes (E1 to E7) and late genes (L1 and L2) besides the upper regulatory region (URR) (Motoyama et al 2004) responsible for viral replication, viral transcription control and cellular transformation. The initial lesions can evolue to cervical cancer mainly by two viral oncoprotein, E6 and E7, due to their ability to immortalize human cell in tissue culture. The synergic effects of these proteins are well known (Moody et al 2010). E7 is capable to inactivate pRB, a protein involved in cell cycle control. The mechanism of carcinogenesis promoted by E7 is highly conserved among HPV types. E7 from HPV58 (3rd most common type of HPV in cervical cancer in East Asia) was found also promoting the proliferation and extending the lifetime of keratinocytes together with down-regulation of pRb/p130 interaction (Zhang W. et al 2010). Papillomaviruses cannot be propagated in tissue culture and attenuated virus cannot be used as vaccine approach due to its oncogenic potential. E7 protein also cannot be isolated from natural lesions because only trace amounts are present (Fernando et al 1999).

Prokaryotic system like *Escherichia coli* remains widely used for recombinant protein production due to its feasibility, well-established protocols, rapid cell growth and low cost of cultivation. However, when milligrams of protein often become necessary, strains with rare tRNA codons has been used (Tegel *et al.* 2010). Recently, a new *E. coli* host KRX that provides protein yields comparable to those of BL21 (DE3) but much higher transformation efficiencies was observed (Schagat et al, 2008). KRX strain allows T7 RNA polymerase-based protein expression controlled by rhamnose promoter that leads to high levels of protein expression related to BL21(DE3)-derived strains (Schagat et al, 2008). Then, this work aimed to clone and induce the production of E7 oncoprotein in KRX expression system in Erlenmeyer's flasks.

MATERIAL AND METHODS

Microorganisms and culture

HPV16 E7 sequence cloned in pET28a (Novagen®) and maintained in *E. coli* BL21(DE3) strain was used to obtain recombinant KRX strain. Bacterial growth and stock were performed in Luria–Bertani (LB) medium (10g.l^{-1} tryptone, 5g.l^{-1} yeast extract, 10g.l^{-1} NaCl) supplement with 30µg.ml^{-1} kanamycin in Erlenmeyer's flasks. Induction with 0.5mM IPTG and 0.1% rhamnose were perfomrd by adding these components into culture medium.

Identification of E7 inserption into KRX strain

BL21(DE3) with pET28-a-E7 was used for colony PCR according to the follow steps: 10µl of suspension bacteria was heated to 56° C for 5 minutes to promote cell lysis and used together with 1µl specific primers (10pmol) (**Table 1**); 6.25 µl GoTaq MasterMix Colorless, ultrapure water was added to 12.5 µl final volume. Amplifications were performed in Gradient Termocycler® (Eppendorf) with the following settings: 95°C for 2 min, followed by 30 cycles at 95°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec, and 72°C for 5 min. The amplified products were observed in 1% agarose gel and the vector transformed in *E. coli* KRX competent cells by heat-shock protocol (Ausubel et al., 1996) and incubated overnight at 37°C in plates with LB media containing 30µg.ml^{-1} kanamycin. Recombinant colonies were selected by Kanamycin-resistance and identified by plasmid extraction using Wizard® Plus SV Minipreps DNA Purification System (Promega). PCR of colonies and nucleotide sequencing was performed to be sure about the integrity of the gene cloned.

DNA sequencing

Conventional PCR was realized, to amplification of vector cloning, using 10 ul of Universal primers (10 pMol), 1X Master Mix Green (Promega), 100ng of DNA and water to complete the reaction. T7 promoter and terminator primers (**Table 1**) was annealing in target plasmid in 370-386 and 26-72 regions. PCR-amplified products were sequenced using 1ul of the same primers (3,2pMol) and fluorescent-based dideoxy sequencing method with DYEnamic™ ET Dye Terminators in automated DNA sequencing system (MegaBACE 750, GE, Life Science

– USA). The sequencing program was 95° C for 20 sec, 50° C for 10 sec, 60° C for 1 min for 25 cycles carried out in MyCycler® Thermocycler (BioRad) MegaBACE 750 Sequence Analyzer (GE Life Science) was used at the endo of the process. Data were submitted to GenBank (http://www.ncbi.nlm.nih.gov/) to be analyzed in BLASTN (Basic Local Alignment Search Tool-nucleotide).

Protein expression

Recombinant bacteria were growth in 50mL of LB media in 250mL flasks incubated at 37ºC and 150 rpm in orbital shaker. When OD600 reached 1.0, around 2 hours, Lac promoter was induced adding 0.5mM IPTG and 0.1% rhamnose. The flasks were maintained at 30ºC and 150 rpm of agitation. Aliquots were harvested in 2 h of culture, to use as control prior to induction. Samples were collected two hours (T2), four hours (T4), six hours (T6) and eight hours (T8) after the induction point for protein analysis. The culture was centrifuge to 3,000 x *g* at 4ºC for 15 minutes and the pellet was suspended in 4mL of breaking buffer (50 mM sodium phosphate pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol). Glass beads were added for corresponding pellet volume for mechanical lyses with four cycles of 30 seconds ice-vortex. Supernatant was harvested at 3,000 x *g* for 20 minutes at 4ºC and analyzed in polyacrylamide gel by electrophoresis. The proteins was denatured with SDS buffer (0.5M Tris-HCl pH 6.8, 10% Glycerol; 10% SDS; 0.08 g/ml de Bromophenol blue; 1% 2-Mercaptoethanol) and incubated for 98ºC for 10 minutes. The proteins analyze was performed in 12.5% and 15% SDS-PAGE condition (Laemmli, 1970) using Rainbow™ Molecular Weight Markers – full-range 12 to 225KDa (GE) as molecular weight marker.

RESULTS AND DISCUSSION

Transformation of recombinant E7 protein in KRX strain

After plasmid extraction and electrophoresis it was possible to visualize plasmidial DNA (**Fig. 1A**) and the PCR screening of *E. coli* KRX.E7 clone resulted in single band (**Fig. 1B**) with around 300pb, confirming the presence of E7 gene in the plasmid. T7 universal primers and E7

primers were used for pET28a-E7.16, showing two different bands of around 400pb and 600pb, respectively (**Fig. 2**). The amplicons were used for sequencing analysis and the best result displayed in MegaBACE Scorecard was submitted to BLASTN, obtaining the score 596; E-value 1e-149; 298/298 (100%) of identify between the cloned sequence and NCBI databank (**Fig. 3**). These results make possible the study of expression of E7 protein in KRX strain of *E. coli*.

Expression of recombinant E7 protein in bacteria

Analyses of total protein production showed that recombinant KRX.pET28a-E7 have similar profile and protein concentration with BL21(DE3), except for sample T2.

KRX containing pET28-a-E7.16 is regulated by a strong rhamnose promoter, which could also lead to a reduction of inclusion bodies formation by reducing the growth temperature for protein induction (Villaverde et al 2003). However, it reduces significantly the expression level of total recombinant proteins (Miladi et al 2010). Our results are in agreement with the comparative analysis of these expression hosts to express TEV protease with IPTG induction being statistically equivalent in their ability although visually the band in SDS-PAGE appears more intense for BL21 (Blommel et al 2007). However, the opposite condition was found by Miladi et al (2010) that reports the change of host as KRX strain results in an increase of 6.5-fold in protein production over BL21(DE3).

12.5% SDS-PAGE showed the target band at 12KDa (**Fig. 4**) in timing T4, T6 and T8. Increasing the gel concentration for improving the resolution of the smallest proteins. A band about 15 KDa was visualized in SDS-PAGE 15% (**Fig. 5**) in protein extract obtained of KRX.pET28a-E7 that could be related to E7 oncoprotein. T7 polymerase band (99KDa) was also visualized, indicating the promoter induction. These data are similar to the reports for other expression systems in *E. coli*. Expression of E7 in *E. coli* C2566 showed a16 kDa band on electrophoresis and reacts with antibodies to E7 protein after purification step (Sharma et al, 2010).

CONCLUSION

We successfully inserted KRX.pET28a-E7 into E. coli KRX strain and expressed this HPV16 oncoprotein, being observed in SDS-PAGE. Further studies are necessary to confirm the E7 protein production by *E. coli* to allow its use in crystallographic and immunological experiments.

Acknowledgment

The authors thank Prof. Dr. Luis Carlos Ferreira from São Paulo University for supplying pET28a containing HPV16 E7 gene.

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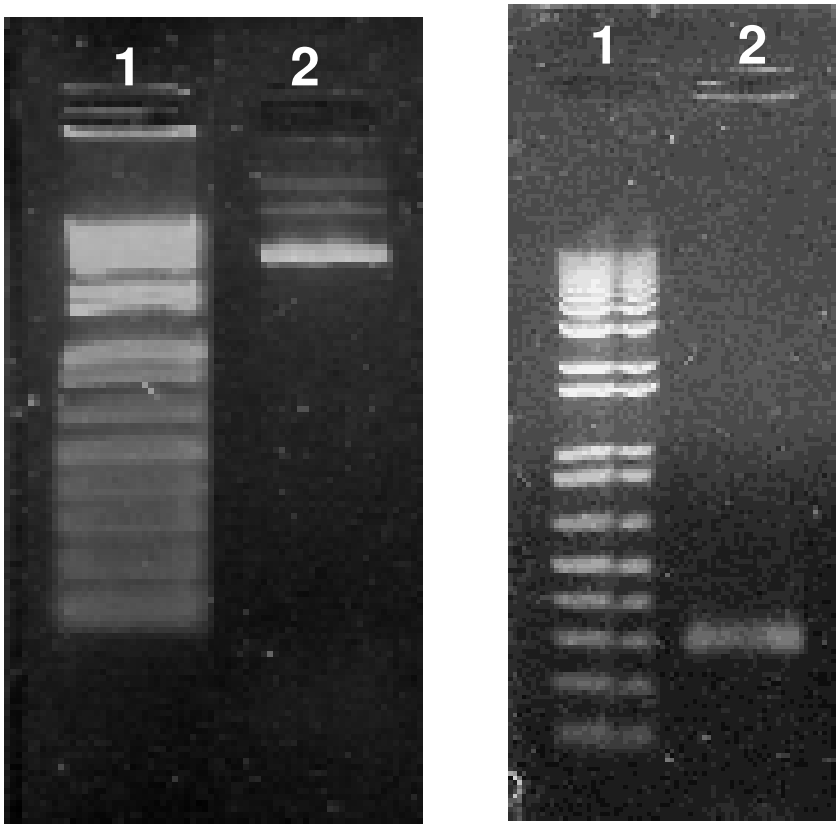


Figure 1A. Agarose of red-faced with ethidium bromide showing pET28-a- E7.16. Lines: (1) Ladder 1Kb Plus, (2) Plasmid extracted (three forms). **1B**. E7 gene (298 pb) amplification, using pET28-a-E7.16 extracted as template and FE7.16 and RE7.16 as specific primers, observed in 1% agarose gel. Lines: (1) Ladder 1Kb Plus, (2) 3µl E7 gene.

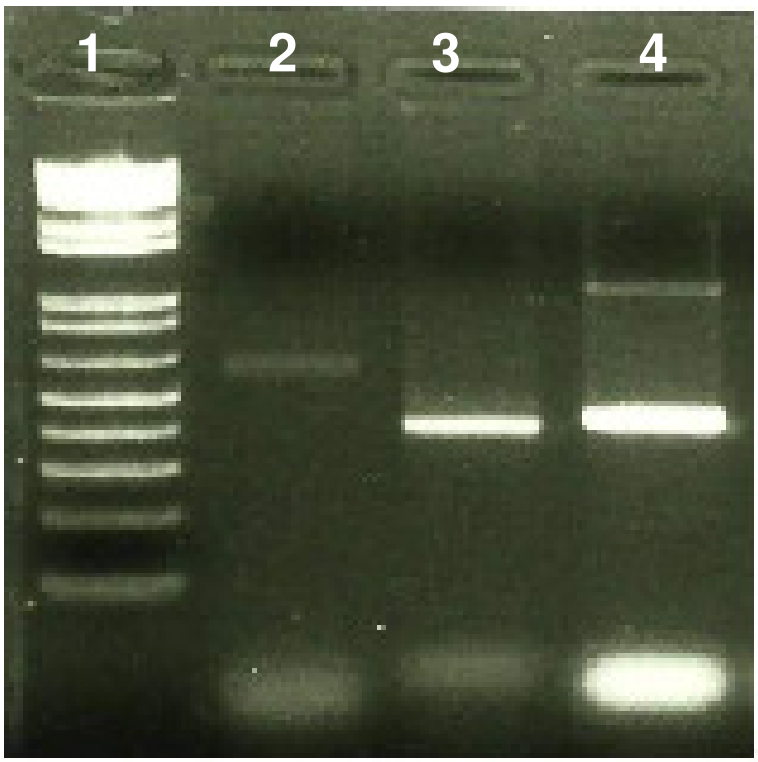


Figure 2. Amplicon resulted of KRX *Escherichia coli* with pET28a-E7.16 PCR using T7 universal primers and specific primers. Lines: (1) Ladder 1Kb Plus, PCR reaction using primers (2) T7 promoter and T7 terminator, (3) T7 promoter and Reverse E7.16, (4) Foward E7.16 and T7 terminator.

Figure 3. Similarity between the GenBank database and nucleotides found after the sequencing of the pET28a -E7.16, plasmid used for recombinant protein production.

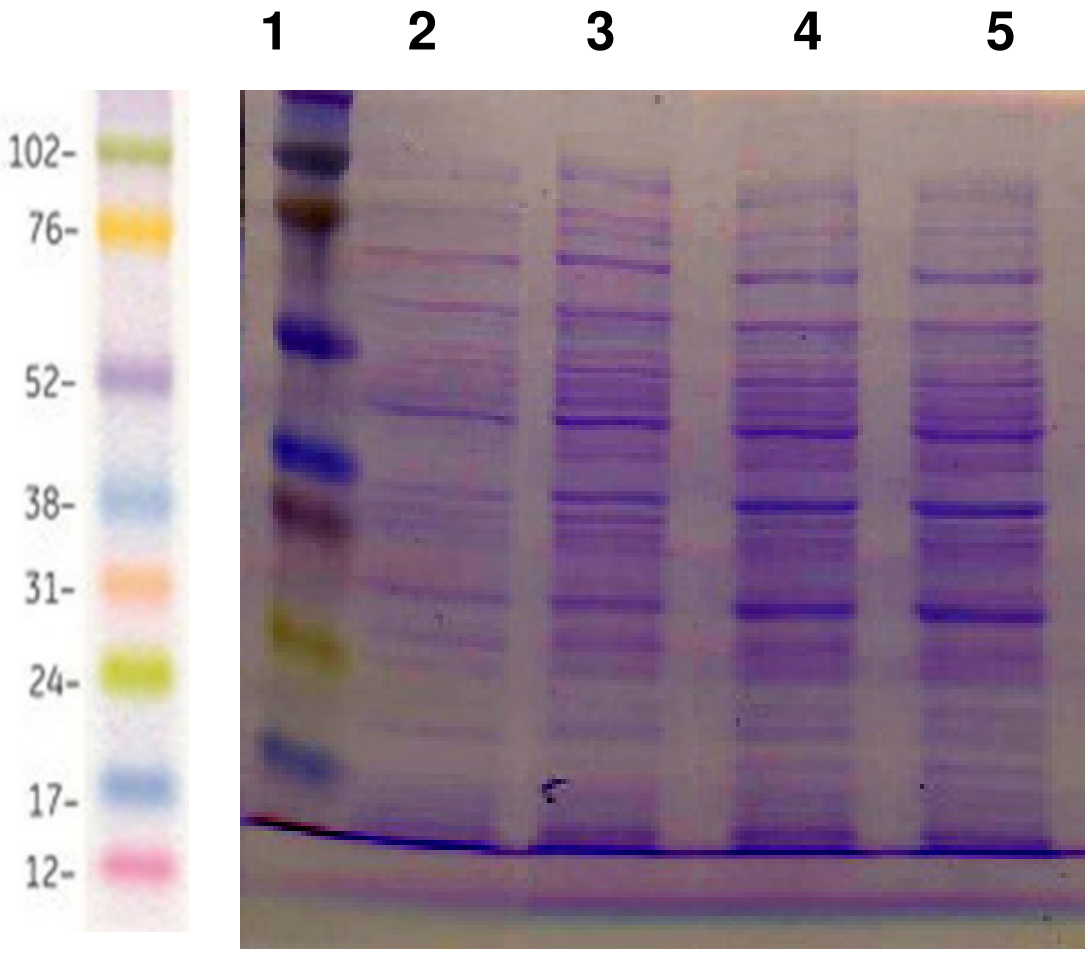


Figure 4. SDS-PAGE 12.5% showed soluble protein extracted of KRX-pET28a-E7. Line (1) protein ladder GE, (2) T2 of KRX-E7, (3) T4 of KRX-E7, (4) T6 of KRX-E7, (5) T8 of KRX-E7.

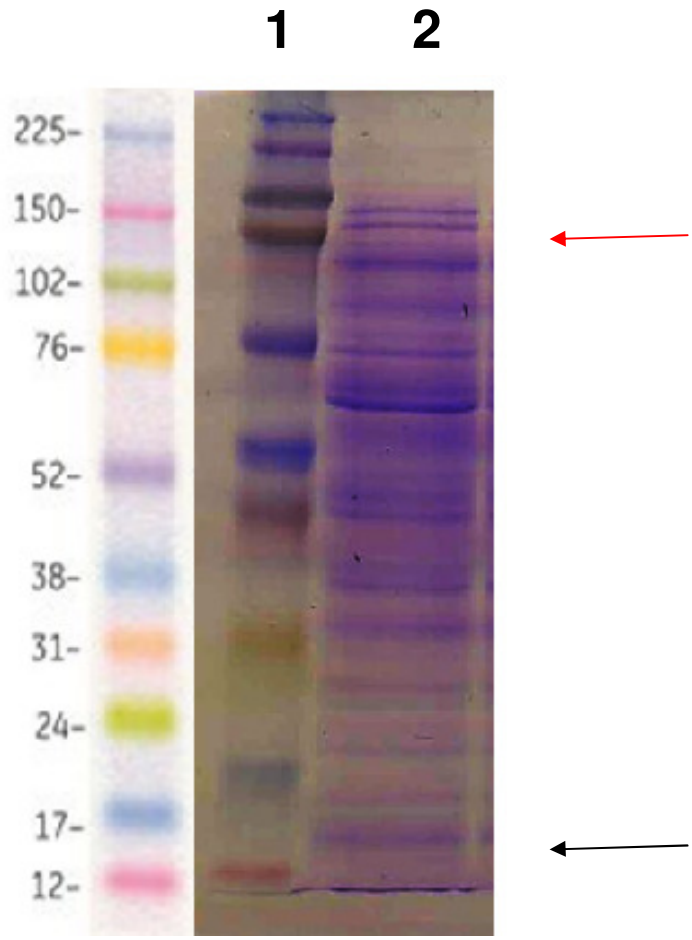


Figure 5. SDS-PAGE 15% showed total protein extracted of KRX-pET28a-E7. Line (1) protein ladder rainbow GE, (2) bacterial lyses with Fast Break reagent. Black arrow has shown the possible region of E7 oncoprotein (~15KDa) and red arrow the possible T7 polymerase (99KDa).

Table 1. Primers used to amplify and DNA sequencing

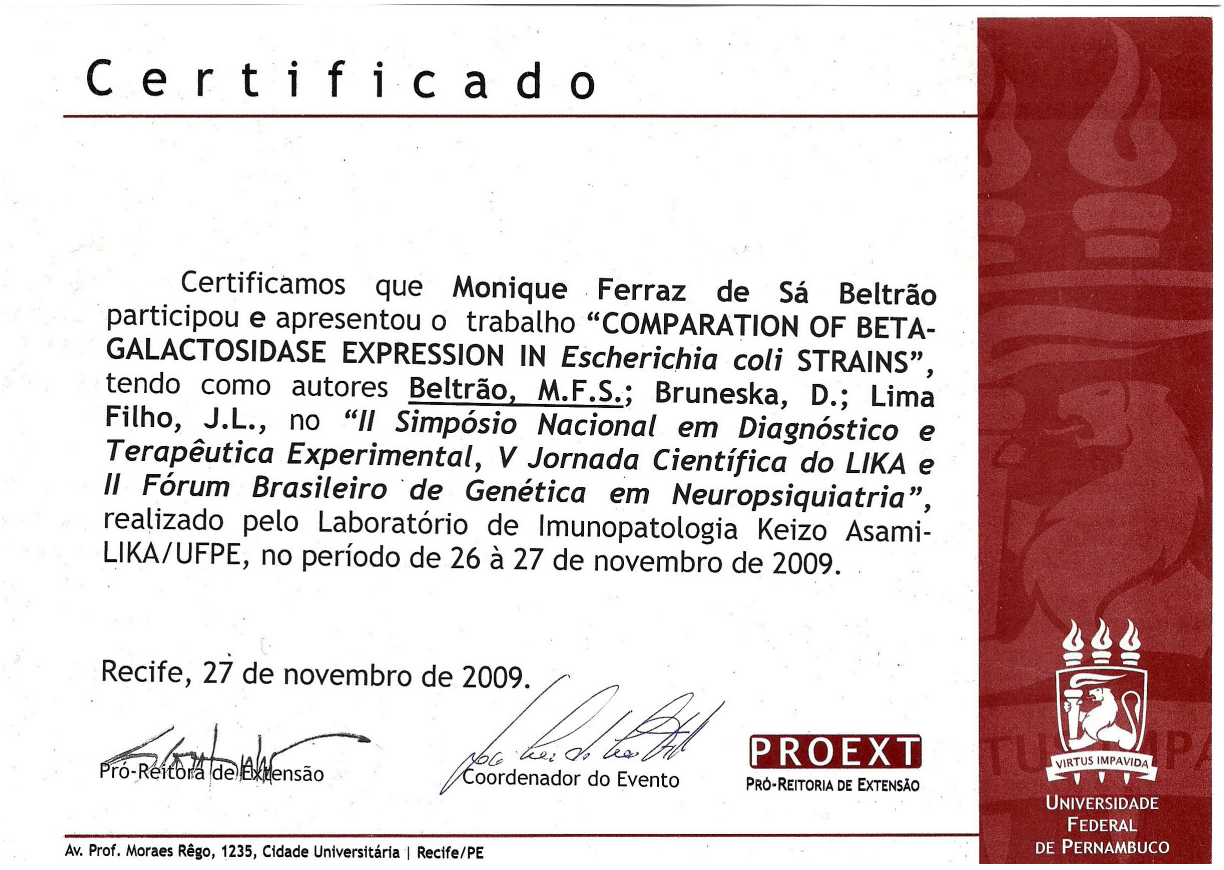
Primer	Sequence 5'-3'	Tm (ºC)
T7 promoter	TAATACGACTCACTATAGGG	50
T7 terminator	GCT AGT TAT TGC TCA GCG G	50
Fw E7.16	ATGCATGGAGATACACCTACA	54
Rv E7.16	TTATGGTTTCTGAGAACAGATG	54

9. INFORMAÇÕES COMPLEMENTARES

9.1. Trabalho apresentado no SINATER 2009

COMPARATION OF BETA-GALACTOSIDASE EXPRESSION IN *Escherichia coli* STRAINS

Beltrão, M.F.S. [1,2]; Bruneska, D. [1,2]; Lima Filho, J.L. [1,2,3]



Certificado

Certificamos que **Monique Ferraz de Sá Beltrão** participou e apresentou o trabalho **"COMPARATION OF BETA-GALACTOSIDASE EXPRESSION IN *Escherichia coli* STRAINS"**, tendo como autores **Beltrão, M.F.S.; Bruneska, D.; Lima Filho, J.L.**, no ***"II Simpósio Nacional em Diagnóstico e Terapêutica Experimental, V Jornada Científica do LIKA e II Fórum Brasileiro de Genética em Neuropsiquiatria"***, realizado pelo Laboratório de Imunopatologia Keizo Asami-LIKA/UFPE, no período de 26 à 27 de novembro de 2009.

Recife, 27 de novembro de 2009.

Pró-Reitora de Extensão

Coordenador do Evento

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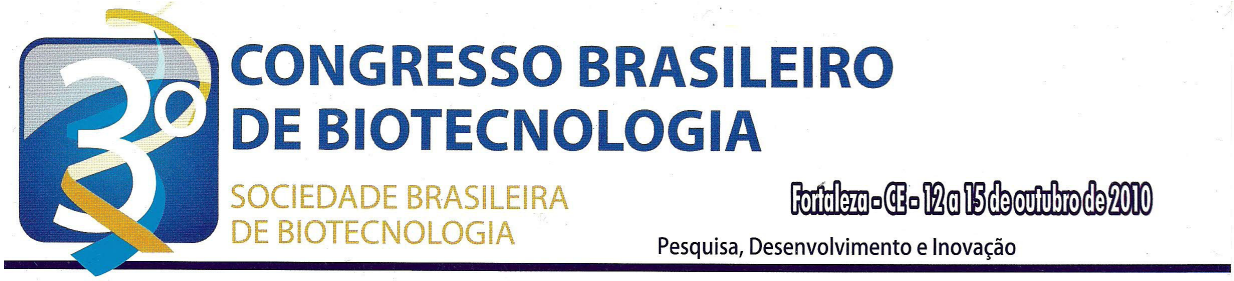
Av. Prof. Moraes Rêgo, 1235, Cidade Universitária | Recife/PE

9.2. Trabalho apresentado no CBBiotec 2010

COMPARISON OF E7 cDNA SYNTHESIS BY Pichia Pastoris IN MEDIUM WITH AND WITHOUT AMINO ACIDS SUPPLEMENT

[1]Monique F. Beltrão, [2]Marcela S. O.Wanderley, [3]Elaine V. M. Souza, , [3]Maíra F. Mafra, [4]Danyelly Bruneska, [4]José Luiz de Lima Filho.



CERTIFICADO

A Comissão Científica do 3º. Congresso Brasileiro de Biotecnologia declara que o trabalho intitulado "Comparison of E7 cDNA synthesis by Pichia pastoris in medium with and without amino acids supplement" autoria de MONIQUE FERRAZ DE SÁ BELTRÃO foi apresentado no 3º. Congresso Brasileiro de Biotecnologia, realizado no período de 12 a 15 de Outubro de 2010, em Fortaleza-CE.

Maria Fatima Grossi de Sá
Presidente da SBBiotec

SOCIEDADE BRASILEIRA DE BIOTECNOLOGIA

Maria Sueli Soares Felipe
Secretária da SBBiotec

10. CONCLUSÃO E PERSPECTIVAS

O HPV pode ser encontrado em diversos sítios de infecção, além dos primeiros sítios corpóreos associados, sendo necessário uma maior atenção dos métodos diagnósticos aplicados, uma vez que o colo do útero ainda é a região mais estudada.

Neste contexto, amostras cervicais de mulheres atendidas na Região Metropolitana do Recife apresentaram alta incidência de HPV16, sendo o variante Europeu o tipo mais predominante em mulheres com lesões genitais. Estas amostras demonstraram um provável HPV16 variante Asiático-Americano, no entanto com ausência de SNP na posição 351 da ORF E6 característica desse variante. Também foi possível identificar uma amostra co-infectada com dois variantes de HPV16 através de regiões de conflito no seqüenciamento do gene E7, caracterizando os tipos Asiático-Americano e Europeu.

A sub-clonagem do gene E7 do HPV16 foi realizada na linhagem KRX de *Escherichia coli,* sendo observada sua expressão nos testes iniciais de indução. No entanto, faz-se necessário o emprego de testes imunológicos para confirmação. A linhagem KRX não correspondeu à expressão esperada de proteína heteróloga. De fato, comparação de três linhagens bacterianas de produção heteróloga da enzima β-galactosidase, realizada previamente, demonstrou que a linhagem ORIGAMI(DE3) de *E. coli* foi a mais indicada por possuir um menor perfil de produção protéica endógena. A utilização do substrato cromogênico X-gal durante o cultivo tornou mais fácil e barato a seleção dos clones .

Estudo epidemiológicos populacionais e de produção de moléculas, como oncoproteínas podem auxiliam na melhor compreensão do mecanismo que faz o HPV induzir a transformação neoplásica nas células infectadas e o desenvolvimento de caminho terapêutico a ser empregado para o câncer cervical na população-alvo.

11. ANEXOS

11.1. Aprovação do Comitê de ética em pesquisa

SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE PERNAMBUCO
Comitê de Ética em Pesquisa

Of. N. º 403/2008 - CEP/CCS Recife, 18 de Dezembro de 2008

Registro do SISNEP FR – 215803
CAAE – 0267.0.172.000-08
Registro CEP/CCS/UFPE Nº 275/08
Título: "Análise do Perfil Epidemiológico de Infecções Causadas pelo Papilomavírus Humano em Mulheres Atendidas pelo Programa Saúde da Família na Cidade de Olinda-PE"

Pesquisador Responsável: Danyelly Bruneska Gondim Martins

Senhora Pesquisadora:

Informamos que o Comitê de Ética em Pesquisa envolvendo seres humanos do Centro de Ciências da Saúde da Universidade Federal de Pernambuco (CEP/CCS/UFPE) registrou e analisou, de acordo com a Resolução N.º 196/96 do Conselho Nacional de Saúde, o protocolo de pesquisa em epígrafe, aprovando-o e liberando-o para início da coleta de dados em 18 de dezembro de 2008.

Ressaltamos que o pesquisador responsável deverá apresentar relatório anuel da pesquisa.

Atenciosamente

Prof. Geraldo Bosco Lindoso Couto
Coordenador do CEP/ CCS / UFPE

José Ângelo Rizzo
Vice-Coordenador do CEP/CCS/UFPE

A
Dra. Danyelly Bruneska Gondim Martins
Laboratório de Imunopatologia Keizo Asami – LIKA/UFPE

Av. Prof. Moraes Rego, s/n Cid. Universitária, 50670-901, Recife - PE, Tel/fax. 81 2126 8588. cepccs@ufpe.br

11.2.1. International Journal of Infectious Diseases

If you have any problem submitting your paper online please contact Annette Fowler at IJID@elsevier.com

The *International Journal of Infectious Diseases* (IJID) is published monthly by the International Society for Infectious Diseases. *IJID*

Please noted as of January 2010 the International Journal of Infectious Diseases will be published online only:

Original articles on infectious disease topics of broad interest. We particularly welcome papers that discuss epidemiological aspects of international health, clinicall reports, clinical trials and reports of laboratory investigations. Original articles should not exceed 5000 words in length.

Reviews on topics of importance to readers in diverse geographic areas. These should be comprehensive and fully referenced. Maximum length 6000 words.

Perspectives are papers that advance a hypothesis or represent an opinion relating to a topic of current interest or importance. They should be fully referenced, and should not exceed 2000 words in length.

Correspondence relating to papers recently published in the Journal, or containing brief reports of unusual or preliminary findings. Maximum length 400 words, one table or figure and a maximum of 10 references.

Case Reports must be carefully documented and must be of importance because they illustrate or describe unusual features or have important therapeutic implications. Maximum length 1200 words, no more than a page and a half in length and a maximum of 1 table or figure. Case reports do not require a structured abstract and should include no more than 5 references.

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11.2.2. Journal of Bioscience and Bioengineering

Instructions to Authors

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Review and publication process

Review process

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Organization and format

Manuscript type

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List all authors when there are 12 or fewer; if more than 13, list the first 10 names followed by "and other 3 authors".Follow the style shown in the examples below.

1. **Takahashi, H. and Honda, H.**: Prediction of peptide binding to major histocompatibility complex class II molecules through use of boosted fuzzy classifier with SWEEP operator method, J. Biosci. Bioeng., **101**, 137–141 (2006).

2. **Ashiuchi, M. and Misono, H.**: Poly-γ-glutamic acid, p. 123–174. In Fahnestock, S. R. and Steinbüchel, A. (ed.), Biopolymers, vol. 7. Wiley-VCH, Weinheim (2002).

3. **Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A., and other 13 authors**: Genome sequence of Bacillus cereus and comparative analysis with Bacillus anthracis. Nature, **423**, 87–91 (2003).

4. **Ohtomo, M., Kimura, K., Watanabe, S., and Toeda, K.**: Production of components containing γ-aminobutyric acid from rice bran by Lactobacillus brevis IFO12005. Seibutsu-kogaku, **84**, 479–483 (2006). (in Japanese)

5. **Kim, M-H., Kino-oka, M., Kawase, M., Yagi, K., and Taya, M.**: Response of human epithelial cells to culture surfaces with varied roughnesses prepared by immobilizing dendrimers with/without D-glucose display. J. Biosci. Bioeng. (2007). (in press)

Examples for references cited in the text:
... as described previously (Ike, M., Abstr. 58th Annu. Meet. Soc. Biotechnol., Jpn., p. 3, 2006).
...were analyzed (Tanaka, S., Ph.D. thesis., Osaka University, Osaka, 2007).
... was observed (Murata, K. and Hashimoto, W., unpublished data).
... by the methods previously described (Kuroda, A., Miyamoto, K., and Ohtake, H., Japanese patent 2008-176450, 2006).

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